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A Comparison of the Compositions of Some Algal Proteins

BY

L. FOWDEN

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Abstract

Representative protein fractions were isolated from *Chlorella vulgaris*, *Anabaena cylindrica*, *Navicula pelliculosa*, and *Tribonema aequale*, which are members of four different algal classes. Quantitative determinations of the amino-acid compositions of these proteins were made, and a comparison of the results shows that a close similarity exists for the proteins of all four species. Subject to certain limitations, the tentative conclusion is reached that the protein complements of all the algae are similar. The compositions of the algal proteins are also compared with those of other plant proteins compiled from data available in the literature.

Introduction

AN extensive literature is available in which the gross chemical composition of many of the algae has been recorded. The analyses have shown that large variations exist in the proportions of the major constituents (protein, carbohydrate, and fat) found in different algal species, whilst the type of carbohydrate and pigment present in the cells also shows some species variation. The accepted classification of this group of plants is in fact largely based on the composition of their pigments and storage products. From the results of more detailed investigations performed on a few algal species it has also been established that the proportions of protein, carbohydrate, and fat present in a single alga are very variable, and depend upon the conditions of growth and age of the culture. A summary of these findings has been given by Fogg (1953). Such investigations have not only provided a wealth of fundamental information concerning the physiology and biochemistry of the algae themselves, but have also provided much of the essential data required for the more recent assessments of the possible practical value of these microscopic plants as sources of food (see Spoehr and Milner, 1948; Meier, 1949; Pearsall and Fogg, 1951; Geoghegen, 1951; Milner, 1951).

The amino-acid compositions of the algal proteins have by comparison received little attention. Mazur and Clark (1938, 1942) examined algae of several different types (mostly marine species), and presented complete amino-acid analyses of their proteins. Although they found that marked differences existed, especially in the percentages of certain of the amino-acids

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(lysine, cystine, methionine, and tyrosine), Lugg (1949) has recently questioned the validity of their conclusions, suggesting that they were based on analyses probably subject to considerable errors due to the use of inadequate methods of protein extraction and hydrolysis. A complete quantitative analysis of the proteins of *Chlorella vulgaris* has also been reported (Fowden, 1951*a*, 1952). In the later publication the amino-acid composition of the protein was shown to remain virtually constant with increasing culture age, although the percentage of protein within the cells steadily decreased. Williams and Burris (1952) have estimated the percentages of some of the amino-acids in four nitrogen-fixing blue-green algae, whilst several other workers have reported the results of qualitative amino-acid assays of other algal proteins (Eny, 1949; Fowden, 1951*b*; Watanabe, 1951; Ericson and Sjoström, 1952).

An analysis of the proteins of four different algae has now been made in order to investigate the extent of any differences in composition between algae of different classes. In presenting the results of this investigation, this paper also compares the compositions of the algal proteins with those of other plant species.

Materials and Methods

1. *Growth of algae*

The algae used were *Chlorella vulgaris*, *Anabaena cylindrica*, *Tribonema aequale*, and *Navicula pelliculosa*. Each of these organisms was available in pure culture, and could be grown in sterile synthetic media. Their forms are comparatively simple, and they could therefore be obtained as nearly uniform suspensions in water. The cultural conditions used for the growth of each alga were as follows:

Chlorella vulgaris, a green unicellular alga (Class—Chlorophyceae), was grown in the medium of Pearsall and Loose (1936) to which Arnon's (1938) A4 trace-element solution was added (1 ml./l.). The initial pH of the medium was between 6·1 and 6·2.

Anabaena cylindrica Lemm., a filamentous blue-green alga (Class—Myxophyceae), was grown on the medium described by Fogg (1949), which had an initial pH between 7·2 and 7·4.

These two organisms were grown in penicillin culture flasks, 500 ml. of medium being introduced into each flask before sterilization. The flasks were maintained at a laboratory temperature of about 20° C. under a low continuous illumination of 150 ft.-candles, and were occasionally gently shaken.

Tribonema aequale, a filamentous yellow-green alga (Class—Xanthophyceae), was grown on the same medium as the *Anabaena*, with the addition of 0·2 g./l. of potassium nitrate, and with ferric citrate replacing ferric chloride.

Navicula pelliculosa, a unicellular diatom, was grown on the medium No. 10 of Chu (1942), with the addition of 1 g./l. of agar.

The two latter organisms were grown in a large rectangular culture-vessel maintained at laboratory temperature and aerated continuously with an

air+5 per cent. carbon dioxide mixture under a constant illumination of about 1,000 ft.-candles.

2. *Harvesting of algae*

The algae were harvested near the end of the period of exponential growth of each culture, the cells being separated from the media using a DeLaval centrifugal separator running at 9,000 r.p.m. The packed cells were removed from the rotor chamber and washed twice by suspending in small volumes of distilled water followed by centrifugation. Each algal sample was then divided into two parts. The smaller portion was dried *in vacuo* and retained for nitrogen assays, whilst the larger portion was resuspended in a suitable volume of borax solution (1·1 g./l.) prior to disintegrating the cells.

3. *Protein extraction and hydrolysis*

The methods used for extracting and hydrolysing the proteins from the algae were essentially the same as those used earlier with *Chlorella* (Fowden, 1951*a*). Briefly the procedure was as follows. The suspension of the organism in borax solution was subjected to a shearing strain of 20,000 lb./sq. in. in a cell-disintegrating apparatus constructed from details given by Milner, Lawrence, and French (1950). The disintegrator had been cooled to 3° C. by storing overnight in a refrigerator. The greater proportion of the cells were disrupted by this treatment and their cytoplasmic contents released into solution. When *Anabaena* is grown under the above conditions the filaments tend to aggregate because the cells produce large amounts of mucilage during growth. It was therefore necessary to break down the aggregates of the alga to give a more uniform suspension in borax solution by shaking vigorously with glass beads before the cells were ruptured. If this procedure was not adopted, the needle valve of the disintegrator easily became blocked. Haemocytometer counts before and after treatment showed that the efficiency of this disintegrating process for all the algae was of a high order, over 90 per cent. of the cells normally being ruptured.

The suspension of broken cells (1 volume) was diluted with more ice-cold borax solution (5 volumes) and an equal volume of an ice-cold ethanol-ether mixture (4 volumes of ethanol to 1 volume of ether) was added slowly with good stirring to the diluted suspension. The mixture was then stored at 3° C. for 1 hour. This treatment disrupted any chloroplasts that remained intact and released their protein into solution, and also prevented interference by fatty materials in the later extraction stages. The suspension was next centrifuged at 500 g. for 30 minutes. The separated solid material, consisting largely of broken cell-walls together with a smaller quantity of unbroken cells, was discarded. The supernatant solution was then adjusted to a pH of about 4·5 with acetic acid, and warmed to 70° C. to flocculate the protein. After cooling, the protein was separated by centrifugation, and then washed successively with dilute acetic acid (pH 4·5), boiling ethanol (twice), dilute citric acid solution, boiling ethanol (twice), and finally with ether. In all cases the

protein fractions obtained were practically devoid of adhering plant pigments, and after drying in air at normal temperatures they were weighed and finely ground to give buff-coloured powders.

Nitrogen estimations on samples of the final protein fractions obtained, and on aliquots of the original suspensions of algae in borax solution, indicated that over 90 per cent. of the total protein present within the cells of each alga was isolated by the method used, if allowance was made for the nitrogen present in those cells remaining intact after the disintegrating treatment and for the non-protein nitrogen content of the broken cells.

The protein fractions were hydrolysed using a mixture of equal volumes of concentrated hydrochloric acid (A.R.) and glacial acetic acid (A.R.) containing 4 per cent. (w/v) of stannous chloride dihydrate. The protein concentration was adjusted to about 10 mg./ml., and the hydrolyses were performed in sealed ampules heated at 100° C. for 24 hours. The proteins were more readily soluble in this mixture of acids than in hydrochloric acid alone, whilst the addition of the reducing agent, stannous chloride, markedly reduced or entirely prevented the humin formation that took place during hydrolysis in its absence. After the completion of hydrolysis the mixtures were evaporated to dryness *in vacuo* at laboratory temperature to remove the volatile acids, and the residues were redissolved in about 5 ml. of distilled water. The stannous chloride was then removed by electrolytic desalting (Consden, Gordon, and Martin, 1947) and the remaining solution again evaporated to dryness. The residues were finally dissolved in distilled water to give solutions containing about 1 mg./ml. of total nitrogen.

4. *Analytical procedures*

Total nitrogen estimations were obtained by the micro-Kjeldahl method of Chibnall, Rees, and Williams (1943) using a Markham still for distillation. The nitrogen present in each alga was separated into soluble non-protein nitrogen and insoluble protein nitrogen fractions by extracting the dried cells three times with 70 per cent. ethanol (1 ml. for each 10 mg. dried material) for 24 hours at laboratory temperature with constant shaking.

Amide nitrogen values were obtained by estimating the ammonia produced during hydrolysis with N—H_2SO_4 for 3 hours at 100° C. using the Nesslerization procedure of Umbreit, Burris, and Stauffer (1945).

Individual amino-acids present in the protein hydrolysates were estimated after separation on paper chromatograms by the quantitative procedure of Fowden (1951*c*) exactly as described previously for *Chlorella* (Fowden, 1951*a*). Cystine and tryptophane assays were made using specific colorimetric procedures; alkaline hydrolysis of the proteins using 5N—NaOH was adopted when tryptophane was to be assayed.

Results

The algae studied differed considerably in the percentages of nitrogen contained within the dried cells, although the proportions of the total nitrogen

present as soluble non-protein nitrogen remained relatively constant for all four species (see Table I for data). Approximate values for the percentage of protein within the dried cells have been calculated by multiplying the

TABLE I

Presenting the Total Nitrogen, Soluble Nitrogen, and Protein Contents of the Dried Algae, and the Nitrogen Content of the Isolated Protein Fractions

	Chlorella vulgaris.	*Anabaena cylindrica.*	*Tribonema aequale.*	*Navicula pelliculosa.*
% total nitrogen in dried cells	4·30	6·51	2·32	2·56
Soluble nitrogen as a % of total nitrogen	10·3	14·0	11·3	11·9
Calculated % protein in dried cells.	24	35	13	14
% nitrogen in the isolated protein fractions	11·5	9·8	11·7	7·6

percentage of insoluble nitrogen by the factor 6·25 (this assumes an average nitrogen content of 16 per cent. for the pure proteins of the algae).

Table I also gives the percentages of nitrogen in the protein fractions isolated. The *Chlorella* and *Tribonema* fractions therefore contained about

TABLE II

Presenting the Amino-acid Compositions of the Protein Fractions isolated from Four Algae. Values expressed as g. Amino-acid Nitrogen per 100 g. of Total Protein Nitrogen

	Chlorella vulgaris.	*Anabaena cylindrica.*	*Navicula pelliculosa.*	*Tribonema aequale.*
Aspartic acid	6·4	6·9	6·4	5·1
Glycine	6·2	5·5	6·1	6·2
Threonine	2·9	5·7	4·2	4·0
Alanine	7·7	6·0	6·5	8·4
Tyrosine	2·8	1·6*	1·9*	3·0
Valine	5·5	7·0	7·5	7·5
Phenylalanine	2·8	2·9	3·4	2·8
Serine	3·3	2·4	4·2	2·4
Glutamic acid	7·8	5·6	4·9	4·6
Leucine	6·1	6·2	7·2	6·4
Isoleucine	3·5	3·9	3·5	4·1
Proline	5·8	5·0	6·2	6·1
Arginine	15·8	11·7*	9·2*	15·9
Histidine	3·3	2·5*	2·8*	3·7
Lysine	10·2	6·6*	8·3*	9·0
Tryptophane	2·1	1·0	1·1	1·8
Methionine	1·4	1·2	1·2	1·4
Cystine	0·2	†	†	†
Amide-N	6·1	8·0	7·1	6·5
Humin	—	+	+	—
Total N accounted for	99·9	89·7	91·7	98·9

— = no humin formation during hydrolysis of proteins.
\+ = slight humin formation during hydrolysis of proteins.
* estimates probably low (see Discussion).
† present in small amounts although not estimated.

75 per cent. protein, whilst the percentage purity of the fractions obtained from *Anabaena* and *Navicula* was lower. At all stages during the isolation of the protein from *Anabaena* the protein had a very gelatinous character, and

TABLE III

A Comparison of the Amino-acid Compositions of Some Plant Proteins. Values expressed as g. Amino-acid Nitrogen per 100 g. of Total Protein Nitrogen

	Algae (present assays).	Potato tuber protein (after Thompson and Steward, 1952).	*Nitrosomonas* (after Hofman, 1953).	Leaf protein Leguminosae (after Lugg, 1949).	Zein of maize (after Bloch, 1945).	Tobacco mosaic virus protein (after Knight, 1947).
Aspartic acid	5·1–6·9	7·9	5	4·7–5·4	3·4	8·6
Glycine	5·5–6·2	6·5	8	—	0·0	2·1
Threonine	2·9–5·7	2·3	4	4·0	2·5	6·4
Alanine	6·0–8·4	6·1	8	—	9·9	4·8
Tyrosine	1·6–3·0	1·9	1	2·3–2·6	5·9	1·7
Valine	5·5–7·5	5·9	7	4·5	3·0	6·6
Phenylalanine	2·8–3·4	6·7	4	2·4	6·6	4·3
Serine	2·4–4·2	3·1	2	—	—	5·8
Glutamic acid	4·6–7·8	8·7	8	6·4–6·7	35·6	6·5
Leucine	6·1–7·4	11·7	3	7·3	3·0	6·0
Isoleucine	3·5–4·1		4	3·6		4·2
Proline	5·0–6·2	2·7	4	—	9·0	4·3
Arginine	9·2–15·9	12·0	8	13·0–14·0	1·6	18·3
Histidine	2·5–3·7	—	3	3·8–4·0	0·8	0·0
Lysine	6·6–10·2	9·6	8	6·4–6·5	0·0	1·6
Tryptophane	1·0–2·1	—	—	1·6–1·9	0·2	1·7
Methionine	1·2–1·4	0·9	2	1·2–1·4	2·5	0·0
Cyst(e)ine	0·2	—	—	1·1–1·3	1·0	0·5
Amide-N	6·1–8·0	14·0	8	5·1–5·3	—	8·0

— indicates no estimate made.

it seems probable that some of the original mucilaginous material adhering to the intact cells was carried through the extraction procedure and appeared as the major impurity present in the isolated protein. The *Navicula* protein fraction may have contained silicates in addition to carbohydrates as impurities, for the extraction procedure was such that silicates present in the intact cells of the diatom could have been precipitated together with the protein when the cell extract was adjusted to pH 4·5. This lower degree of purity led to a slight production of humin during the hydrolysis of the *Anabaena* protein fraction, and to a more marked precipitation of humin during the hydrolysis of the *Navicula* protein sample. No humin formation occurred during the hydrolysis of the *Chlorella* and *Tribonema* fractions, and the final hydrolysates were a pale yellow colour.

Table II gives the amino-acid compositions of the proteins isolated, the nitrogen present in each amino-acid being expressed as a percentage of the total nitrogen content of the proteins. The values quoted represent the means of three determinations in all instances, other than the values for cystine and tryptophane which are the means of duplicate determinations. Although cystine was present in low concentrations in all the hydrolysates, insufficient material was available for three of the algae for quantitative estimates to be made.

Table III presents a comparison of the amino-acid compositions of the algal proteins examined here with the protein compositions of some species of other plant orders that have been recorded previously by other workers.

Discussion

This comparative study of the proteins of some algae was undertaken in order to establish whether the same types of protein were present in the different species. The basis of comparison used here, namely the amino-acid compositions of the proteins, although very useful, is, however, subject to certain limitations. The protein fractions isolated from the algae and later analysed cannot necessarily be regarded as being individual proteins. The fractions were in fact probably mixtures of all the different proteins that may constitute the protoplasm of cells undergoing active growth, since it was shown that the isolated proteins represented over 90 per cent. of the whole protein within the cells. The analyses then only provide an average amino-acid composition of what may be termed the 'bulk' protein of each species and can give no information regarding the nature of individual proteins. A second limitation to the interpretation of the results is that it is possible for two or more proteins to possess quite different types of biological activity due to the different arrangements of the amino-acid residues within the protein molecule, although the proteins themselves may have very similar amino-acid compositions.

The experimental data given in Table I show that the total nitrogen and protein contents of the four algae examined varied widely, the values found for *Anabaena* being nearly three times as large as those for *Tribonema*. In certain algae, where only small amounts of protein are present, the protein components may be more biologically active and therefore of a somewhat different type to the proteins present in the algae possessing high nitrogen contents. In higher plants a high nitrogen content of the tissues has often been found to be associated with an abundance of amide nitrogen, especially in the form of uncombined glutamine and asparagine. No determinations of uncombined amides present in the algae were made, but there is little evidence for the possibility that a high nitrogen status in a particular alga is associated with an abundance of combined amide nitrogen in its protein components. The amide nitrogen proportions of the four algal proteins show only small differences. *Anabaena* protein was found to contain only slightly more amide

nitrogen than the proteins of the other algae (see Table II), and *Chlorella*, although possessing the second highest total nitrogen content, showed the lowest amount of combined amide nitrogen.

The results presented in Table II also show that the proportions of most of the individual amino-acids present in the algal protein fractions are similar for those species examined. Some of the amino-acids (glycine, phenylalanine, leucine, isoleucine, methionine, and proline) are present in each of the four proteins in approximately equal amounts if allowance is made for normal experimental errors. Significant differences between the percentages of certain amino-acids (arginine, histidine, lysine, tyrosine, threonine, serine, and glutamic acid) are apparent. The values obtained for the arginine, histidine, lysine, and tyrosine contents of the proteins isolated from *Anabaena* and *Navicula* are all lower than the corresponding values determined for *Chlorella* and *Tribonema* proteins. These four amino-acids, together with the sulphur-containing amino-acids and tryptophane, are partially or wholly destroyed by acid hydrolysis of proteins contaminated with appreciable amounts of carbohydrate material, and a proportion of the amino-acid nitrogen is converted to humin nitrogen (Lugg, 1939). It would then appear probable that the differences in the values obtained for the basic amino-acids and tyrosine may be largely due to the destruction of a proportion of these amino-acids during the hydrolysis of the *Anabaena* and *Navicula* protein fractions, when some humin formation was observed to occur. Differences that may be regarded as real then only exist for the three amino-acids threonine, serine, and glutamic acid. Williams and Burris (1952) have found a similar distribution of amino-acids in the proteins of four blue-green algae, although they did not determine the percentages of all amino-acids present.

Subject to the limitations set out above, these findings would appear to justify the conclusion that the proteins present in algae are of a fairly uniform character and not subject to wide species variations like those encountered when pigment distribution is considered. It may be possible to test this tentative conclusion further by using electrophoretic methods to investigate the intact proteins of the algae; future experiments are planned to assess the usefulness of these techniques for such an investigation.

The data given in Table III indicate that this general similarity in protein composition is not confined only to the algae. The compositions of the 'bulk' proteins of leaves (Leguminosae), of potato tubers, and of the micro-organism *Nitrosomonas* all show a close similarity to those found for the algae. As with the algae, each of these materials probably contains a number of individual proteins responsible for maintaining the complex pathways of metabolism within the tissues, and so the analyses may do no more than indicate that the average amino-acid compositions of all the proteins of each species are similar. The compositions of such 'bulk' proteins (probably consisting largely of albumin and globulin types of protein) are also compared in Table III with the compositions of proteins which are either homogeneous or composed of simple mixtures of a limited number of very similar proteins. Examples of

the latter type of protein quoted in the table are zein (a prolamine type of protein which is the main reserve protein of maize seeds) and the protein present in tobacco mosaic virus. Large differences occur between the amounts of some amino-acids (glycine, arginine, histidine, lysine, glutamic acid, and methionine) present in these proteins and the amounts found for the more complex proteins discussed earlier.

When the algal proteins are considered with regard to their possible nutritional value, they are seen, like many other plant proteins, to be deficient particularly in the sulphur-containing amino-acids, cystine and methionine.

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The Effect of Manganese on the Assimilation and Respiration Rate of Isolated Rooted Leaves

BY

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With Plate XV and four Figures in the Text

Abstract

The effect of manganese on carbon assimilation, respiration, and translocation has been studied using isolated rooted potato leaves and small potato plants. Methods are described for the rooting and culture of the leaves and plants. It was found that normal potato leaves rooted readily when treated with α-naphthaleneacetic acid (2 p.p.m.), but that very few of the manganese-deficient leaves produced roots, the critical level being about 15 p.p.m. manganese on a dry weight basis. The growth of isolated deficient leaves was also much less than that of control leaves, but in no case did characteristic manganese-deficiency symptoms develop, although the manganese level had fallen below that of leaves which showed symptoms when attached to the plant. A marked difference in net assimilation rate was found between leaves which had a high or low manganese content at the time of rooting. The addition of manganese after rooting to low manganese leaves did not, however, cause an increase in assimilation rate, although the manganese content of the leaves had been raised to that of the control leaves. Manganese was shown to have only a small effect on respiration, higher respiration rate being consistently associated with a higher manganese content; the addition of manganese to 'deficient' leaves did not cause any increase in respiration. No effect of manganese on translocation was detected.

Introduction

THE importance of manganese in metabolism of plants is now established but its function is still obscure. Bertrand, in 1897, was apparently the first to have considered the possibility of a physiological function of manganese in plants.

Since manganese is needed in such small concentrations it is generally assumed that it takes part in enzymatic reactions. Bertrand (1897) claimed that the activity of laccase and other oxidases depended on their manganese content. Later work has shown that laccase is a copper protein containing no traces of manganese (Keilin and Mann, 1939, 1940; Tissières, 1948), and according to Mann (1949), 'No manganese protein with oxidase properties

[1] Part of a thesis submitted to the University of London for the Ph.D. degree in 1952.

has yet been isolated.' There is, however, considerable evidence that manganese activates certain enzyme reactions and Hewitt (1951) has reviewed this subject.

If manganese takes part in enzyme reactions it may obviously influence the metabolic processes of the plant in many ways, e.g. it may affect photosynthesis, respiration, or translocation. In this paper the effect of manganese on these processes was studied, chiefly by the use of isolated rooted potato leaves as a source of experimental material.

The use of isolated rooted leaves in physiological experiments was suggested by Gregory and Samantarai (1950). They claim that rooted leaves have the following advantages over detached leaves or whole plants:

(*a*) Physiological experiments (e.g. on assimilation, translocation, or respiration) may be prolonged without deterioration of the material.
(*b*) Changes in metabolism in 'deficient' leaves may be followed after supplying the deficient element through the root system.
(*c*) Mobilization of nutritive factors from the rest of the plant is avoided.

The possibility of a different response in behaviour of isolated rooted leaves from those attached to the plant must be borne in mind, and many of the experiments that will be described later have been duplicated using whole plants.

Experimental Procedure

To provide material potato plants were grown in sand and in water cultures.

Water-supply

Tap-water was passed through two 'Permutit' units in series and then through a Pyrex glass column (1 in. diameter, 24 in. long) containing 'Biodeminrolit F' resin, which was renewed each month. Later the use of the resin was discontinued, analyses having shown no decrease in manganese content of the water after passage through the resin. Large amounts of carbon dioxide are liberated in the demineralization process, which apparently caused rapid exhaustion of the resin. This is perhaps not surprising as during the season 50–100 gallons of water were used per week.

Total solids of the purified water as estimated by evaporation were found to be 2 p.p.m., of which less than 0·001 p.p.m. was manganese. Thus a high quality water was obtained without the use of the resin column.

Nutrient solution

A modified Shive and Robbins (1938) nutrient solution was employed of the following composition:

Manganese-deficient nutrient solution

K_2HPO_4	0·0025 M	Fe as ferrous sulphate ('Spec pure')	3 p.p.m.
$Ca(NO_3)_24H_2O$	0·0045 M	Zn as zinc sulphate	0·06 p.p.m.
$MgSO_4.7H_2O$	0·0023 M	Cu as copper sulphate	0·06 p.p.m.
$(NH_4)_2SO_4$	0·002 M	B as boric acid	0·5 p.p.m.

The same solution to which 0·25 p.p.m. manganese as sulphate was added served as control.

Stock solutions of the nutrient salts were prepared, using the precipitation technique of Stout and Arnon (1939). These stock solutions were diluted with the 'Permutit' water to give the required concentrations.

Culture methods

The sand-culture plants were grown in 10-in. flower-pots coated internally with three layers of Bitumastic paint. The drainage-holes were plugged with acid-washed glass wool and covered with a watchglass. The pots were filled with acid-washed sand that had been leached with water and nutrient solution to remove excess acidity. The plants grown in water culture were first raised in sand and later transferred singly to the water-culture vessels. One-gallon glazed urn-liners were found to be of a satisfactory quality and capacity for the growth of single plants. Ten-inch flower-pots (5 litres capacity) made watertight with cement and painted internally with three coats of 'Bitumastic' and finally coated with 'Bituros' were also found suitable. The pots were allowed to weather for a fortnight before use. Two-litre capacity Pyrex glass beakers, enclosed in black paper to exclude light, also proved satisfactory for smaller plants.

The cultures were not aerated, preliminary experiments having shown that provided the solution was changed once a fortnight there was no advantage in aeration. Street, Kenyon, and Watson (1946) have, however, stressed the need for aeration for the satisfactory growth of potatoes in culture solutions. It is possible that the divergent result found is due to the difference in composition of the nutrient solutions used. The pH of the culture solution was tested at intervals with a glass electrode and was maintained between pH 6·8 and 7·0.

Symptoms of manganese deficiency

In both sand and water cultures symptoms of manganese deficiency were observed 7–8 weeks after planting; these usually appeared first on the younger leaves and later on some of the older ones.

Two types of symptoms were noted: (1) numerous small black spots on young leaves; (2) large brown spots on older leaves that previously had been green.

A slight chlorosis was also evident on the apical leaves of the deficient plants.

More than 1,000 plants were raised during the growing season, and in general deficiency symptoms appeared first on the younger leaves. However, in one batch of 50 plants grown in sand cultures approximately 80 per cent. of the plants developed large brown spots on the basal leaves, the tip leaves at this time (June 7) being green with no trace of symptoms. By June 12 the characteristic fine spotting was visible on some of the apical leaves. Many of

the old leaves had withered by this time. Such development of symptoms on the basal leaves was an isolated case, and the cause is unknown. Many workers have shown that manganese-deficiency symptoms first appear on the younger leaves—of peas by Piper (1941), tomatoes by Lyon, Beeson, and Ellis (1943), citrus by Haas (1932), tung by Reuther and Burrows (1942), and tobacco by Swanback (1939). Wallace (1951) has, however, stated that 'grey-speck' of oats usually appears on the older leaves, and that in apples, plums, and raspberries young leaves of terminal shoots may not be so severely affected as older leaves.

Within 2 weeks of the appearance of deficiency symptoms, the manganese-deficient plants had ceased to grow. The apical leaves and shoots had withered, brown spots being present on the 'wings' of the stems of the plants most severely affected. Necrotic areas were also present on the shoots bearing the flowers leading to a severe 'topple'. The control plants were much larger, the leaves a dark green colour, and the flowering profuse.

The root systems of the deficient plants were somewhat less developed than the controls but remained perfectly healthy.

About a month after the appearance of symptoms, the early shoots of the deficient plants had died. Meanwhile numerous young basal shoots had formed which at first were green, but later also developed typical symptoms. It thus appears possible that there is a migration of manganese from the old dying shoots to new shoots arising at the base of the plant, or that buds already contain manganese which is not lost while the buds are dormant.

Detached sprouts

Potato tubers that have been sprouted in the light for a period develop small swollen shoots, which if detached and placed in a suitable medium, root readily within a few days. Rooting of these sprouts was found to be satisfactory both in water and sand cultures. When detached sprouts were placed in a manganese-free nutrient solution deficiency symptoms were produced 3 weeks from planting.

Detached buds

Leaves with axillary buds attached were removed from plants and rooted in sand or water cultures by a technique to be described later. After rooting the leaves were removed, and the rooted buds transferred to culture solutions with and without manganese.

When rooted detached buds were planted in a manganese free solution, deficiency symptoms were apparent within 10 days, whereas in the control culture solution buds developed normally. By these three methods of propagation using whole tubers, detached sprouts, and rooted buds, control of plant size, manganese content, and of time of appearance of deficiency symptoms was achieved.

Rooted Leaves

Although the propagation of certain plants by means of leaf cuttings is an established horticultural practice, the use of isolated rooted leaves in physiological experiments with trace elements apparently has not been attempted previously.

Rooting of potato leaves

Factors influencing the regeneration of roots and shoots in leaf cuttings of many species of plants have been investigated extensively (Swingle, 1940; Thimann and Poutasse, 1941; Gregory and Samantarai, 1950).

Knight (1816), Kupfer (1907), and Isbell (1931) investigated regeneration in isolated potato leaves. They found that rooting was poor when axillary buds were removed from the leaf cuttings, and in general no shoot or tuber was formed, although an enlargement of the end of the petiole into a tuber-like organ containing starch was sometimes noted. If the axillary bud was retained either a shoot or tuber was produced. No indication of root formation with this type of leaf-bud cutting was given.

Application of hormones in widely differing concentrations have been shown to stimulate rooting of isolated leaves. In preliminary experiments with potato leaves, tests were carried out to find the most suitable hormone and concentration for rooting in this species.

The fourth leaf from the apex of normal potato plants was used. Isolation of the leaves took place on June 2, 1949, and they were immediately treated with various hormone solutions by immersing the cut petiole for 24 hours, in the light. The following hormones were used:

	Concentration in p.p.m.		
	Low.	Medium.	High.
β-Indolyl acetic acid (IAA) . . .	2·5	5	10
β-Indolyl butyric acid (IBA) . . .	1	2	4
α-Naphthalene acetic acid (NAA) . .	0·5	1	2

Sets of fifteen leaves were used in each treatment.

After treatment the petioles were washed and the leaves planted. Rooting was first observed 10 days after treatment; 30 per cent. of the cuttings under all treatments rooted. The best response was obtained with NAA at 2 p.p.m., followed by NAA at 0·5 p.p.m. and IBA at 4 p.p.m. In subsequent experiments NAA at 2 p.p.m. was used as a standard dip for a period of 24 hours.

Investigations on the best type of rooting medium were also made. The results were equally good in sand, a mixture of peat and sand, vermiculite, tap-water, or culture solution. In practice sand or culture solutions were usually employed.

Effect of leaf size and position on rooting response

Leaves of various sizes (49 in all) were removed on July 8, 1949, weighed,

and treated with NAA. The treated leaves were set in a peat soil mixture in a propagating frame. Table I shows the percentage of rooting after 2 months; the leaves are divided into three size groups.

TABLE I

Relation of Leaf Size to Percentage Rooting

Weight group (g.).	No. of leaves taken.	% rooted.
0–2	24	25
2–4	17	35
4–6	8	25

Leaves of all series produced roots, and medium-sized leaves were the most responsive.

In a second experiment (May 1, 1951) the effect of leaf position on rooting of leaves taken from high and low manganese plants was compared. The plants were grown in sand cultures and fed with high and low manganese nutrient solutions. Both series of plants were of a similar size, and it was possible to compare corresponding leaf positions.

Leaves were chosen at the 4th, 5th, 6th, 7th, and 8th nodes from the apex on high and low manganese plants, at a time when there were approximately ten expanded leaves on the plants.

Table II shows the percentage of rooting both in high and low manganese treatments at the five different leaf positions.

TABLE II

Rooting at Different Leaf Positions

	High Mn.		Low Mn.	
Leaf number from apex.	No. of replicates.	% rooted.	No. of replicates.	% rooted.
4	19	100	25	76
5	18	89	24	42
6	17	65	23	48
7	12	67	19	52
8	10	80	12	58

It will be seen that the position of the leaf on the plant over the range studied did not greatly influence rooting response and, in general, manganese-deficient leaves rooted less readily.

Individual leaves showed widely different reactions. Many leaves remained unrooted but at the end of 2 months were green and healthy, as Knight (1816) found. Others produced swellings at the base of the petiole some of which also produced root systems. The cut, swollen base showed the presence of starch when tested with iodine. A third group produced a basal callus, which was pared on two occasions, but this did not lead to root production. Pl. XV, *a* shows the various conditions of the cuttings.

Rooting of manganese-deficient leaves

A series of experiments was carried out to study the effect of manganese deficiency on the rooting response of leaves.

On August 3, 1950, thirty leaves were selected from shoots, of which some leaves showed typical manganese-deficiency symptoms. These were taken from plants grown on the manganese-deficient soil in the lake-bed delta at East Malling.

The leaves were treated as described with NAA 2 p.p.m. and planted in manganese-free sand in 3-in. pots coated internally with bitumen, and supplied with water from the 'Permutit' apparatus. Within 3 weeks 80 per cent. of these leaves had rooted.

Three weeks later a second experiment was carried out. By this time marked manganese-deficiency symptoms had appeared on many leaves of the lake-bed plants, especially on the younger leaves. Forty leaves showing various degrees of spotting were selected from the plants and forty leaves from normal plants were used as controls. Within 4 days of treatment many of the severely spotted leaves had withered and died. After 2 weeks only twelve of the less severely spotted leaves had rooted and survived; at this time 100 per cent. of the controls had rooted.

In a third experiment (May 1, 1951) leaves were taken from comparable positions on high and low manganese water-culture plants, and given the usual hormone treatment and culture technique. Within a fortnight rooting commenced in both series. Some of the deficient leaves were yellow, and many had died by this time. Rooting responses were as follows:

High Mn.		Low Mn.	
No. of replicates.	% rooting.	No. of replicates.	% rooting.
76	81·5	103	58·2

The data indicate that there was a difference of 38 per cent. in rooting in favour of the high manganese leaves. It was concluded that leaves showing severe deficiency symptoms fail to root, but that leaves with slight symptoms from deficient plants can be satisfactorily rooted.

A wide variation in manganese concentration was found in leaves which had rooted, varying from about 100 p.p.m. to 10 p.p.m. on a dry weight basis. There were, however, very few rooted leaves containing less than 15 p.p.m. manganese. A concentration of manganese round about 15 p.p.m. therefore seems to be the critical level for rooting response.

Symptoms of manganese deficiency in rooted leaves

As already stated, leaves of quite a low manganese content sometimes formed roots, even when they showed deficiency, but it is important to note that after rooting no further development of symptoms occurred on such leaves already showing signs of manganese deficiency, nor in any case did normal leaves develop symptoms when cultured without manganese. Thus it would appear that the development of deficiency symptoms is conditional

upon contact of the leaf with the rest of the plant. There are two possibilities for explaining this striking fact: either manganese is withdrawn from the leaves attached to the plant by translocation, or some substance responsible for the development of symptoms enters the leaf from the rest of the plant. The second possibility has already been suggested by Gerretsen (1937) in connexion with 'grey-speck' of oats. The matter will be considered again later in the paper.

Growth of isolated leaves

(*a*) *High manganese leaves.* The leaf areas of the leaflets of high manganese rooted leaves were estimated by means of a leaf print method. Sixty rooted leaves were selected and random samples of twenty were taken at weekly intervals, and their leaf areas determined. The results are shown below:

Mean leaf area (sq. cm.).		
July 18.	July 25.	August 3.
23·8±1·1	28·1±2·5	35·0±4·4

There was a 47 per cent. increase in leaf area during the 2-week period.

(*b*) *Manganese-deficient leaves.* In a similar experiment to that described above, the leaf areas of the deficient leaves were determined in three representative samples of twenty leaves each at weekly intervals:

Mean leaf area (sq. cm.).		
July 18.	July 25.	August 3.
29·2±1·3	25·5±1·8	28·3±2·3

In contrast to the normal leaves, no increase in leaf area was observed. It was concluded that isolated potato leaves appear to have only a limited capacity for further growth, and it has been observed by Gregory and Samantarai (1950) that rooted leaves never attain the size they would have reached if left on the parent plant.

Culture methods for rooted leaves

The hormone-treated leaves on rooting were usually transferred to Pyrex beakers (600 c.c.) containing nutrient solution similar to that in which the parent plants were grown. The solution was renewed weekly. The beakers were enclosed in black paper and the rooted leaf supported in a split waxed cork. No aeration was found necessary. The appearance of typical high and low manganese leaves 6 weeks after rooting is shown in Pl. XV, *b*. It is evident that deficient leaves show less branched roots; the length, however, is the same in both. It appears possible therefore that the activity of the apical meristem of the root is unaffected by manganese, whereas the lateral root development is reduced in its absence. The roots were white and healthy in both treatments.

After a further 3 weeks a pronounced interveinal chlorosis became apparent on the 'normal' leaves, giving them an unhealthy appearance. A similar chlorosis together with a marked inward curling of laminae were found in the

deficient ones, but no other characteristic manganese-deficiency symptoms occurred.

The chlorosis in the normal leaves suggested some nutrient deficiency, and this was tested by an injection technique. The following elements were tested: iron, manganese, zinc, copper, nickel, boron, nitrogen, phosphorus, potassium, magnesium, and calcium, but in no case was a response shown to injection.

It is thus apparent that under the above cultural conditions rooted potato leaves retain their healthy appearance for about 1 month, and all the experiments to be described in later sections of this work were carried out within this period.

Method of manganese estimation

The manganese content of the experimental material was determined by the 'tetrabase' method, as first used by Tillmans and Mildner (1914), and

TABLE III

Variation in Weight and Manganese Level of Distal and Proximal Leaflets taken from Random Single Leaves

	High manganese leaves.					
	Distal leaflets.			Proximal leaflets.		
Sample No.	Dry wt. (g.).	Mn conc. (p.p.m. D.W.).	Mn content (μg.).	Dry wt. (g.).	Mn conc. (p.p.m. D.W.).	Mn content (μg.).
1	0·0628	120	7·54	0·0482	135	5·77
	0·0518	135	7·03	0·0344	163	5·60
2	0·0257	88	2·25	0·0278	79	2·20
	0·0249	76	1·90	0·0291	67	1·95
3	0·0337	100	3·34	0·0306	123	3·75
	0·0348	81	2·80	0·0267	127	3·40
4	0·0332	84	2·78	0·0295	93	2·76
	0·0438	100	4·39	0·0251	80	2·00
5	0·0184	90	1·65	0·0212	86	1·82
	0·0188	97	1·92	0·0264	86	2·27
6	0·0374	183	6·83	0·0151	166	2·50
	0·0292	185	5·40	0·0230	84	1·93
Mean	0·0345	112	3·99	0·0277	107	3·00
	Low manganese leaves.					
7	0·0331	23	0·74	0·0186	25	0·46
	0·0348	24	0·82	0·0260	22	0·57
8	0·0385	31	1·20	0·0393	30	1·19
	0·0402	32	1·27	0·0401	29	1·15
9	0·0532	23	1·25	0·0362	23	0·83
	0·0484	21	1·04	0·0278	27	0·75
10	0·0289	33	0·95	0·0304	31	0·93
	0·0290	35	1·01	0·0305	35	1·06
11	0·0514	29	1·49	0·0308	32	0·99
	0·0390	31	1·21	0·0216	32	0·69
12	0·0204	34	0·69	0·0147	29	0·42
	0·0270	25	0·67	0·0141	28	0·40
Mean	0·0370	28	1·03	0·0275	29	0·79

modified by Nicholas (1946). Certain further modifications were, however, made, and the method is accordingly described.

About 20 mg. dried plant material was ashed at 420° C. in a 7-c.c. Pyrex beaker, cooled, then fumed down with two drops of redistilled hydrochloric acid at 100° C.; cooled again and extracted with 2 c.c. of the acetate buffer (Morgan's solution) for 1 hour. The extract was then transferred to a volumetric flask and made up to an amount suitable for the particular material with distilled water. A suitable aliquot of this solution was made up to 10 c.c. with Morgan's solution, mixed well, and 0·5 c.c. potassium periodate solution added, mixed, and 0·1 c.c. tetramethyldiaminodiphenylmethane added. The blue colour which develops almost immediately was measured in an EEL colorimeter calibrated daily against standard solutions of manganese sulphate.

Manganese analysis of experimental material

The agreement between opposite leaflets taken from distal and proximal positions on a single compound leaf is shown in Table III. The data show that manganese status of a leaf may therefore be assessed with some confidence from the analysis of a single leaflet, and this method was found of value in determinations on rooted leaves in which only a few leaflets were available, when changes in the content during the course of an experiment were studied.

The variation in manganese content of the successive leaves of deficient and control plants was determined by analysis. The leaves were numbered from the apex downwards. The 8th leaf was in each case about half-way down the stem. The analyses were carried out at the end of May 1950 and are shown in Table IV.

TABLE IV

Manganese Content of Successive Leaves on Deficient and Normal Plants

	Deficient.		Normal.	
Leaf No. from apex.	Mn p.p.m.	Symptoms.	Mn p.p.m.	
1	4·7	Severe spotting	33·8	
2	7·7	Moderate: fine and large spots	29·2	
3	10·2	Slight large spotting	27·6	
4	11·6	Severe large spotting	33·1	No
5	12·1	Moderate spotting	49·6	symptoms.
6	12·5	Slight spotting	54·1	
7	14·6	Few scattered large spots	64·7	
8	18·9	„ „ „ „	96·3	

The leaves of the control plants were all dark green and without symptoms. There is a marked gradient in manganese content decreasing in successive leaves from base to the apex of the plant; as development proceeds the manganese content of the individual leaves decreases, whether they are supplied with manganese or not.

Similar gradients have been found in citrus by Haas (1932), in tomato by

Lyon, Beeson, and Ellis (1943), and in tung by Drosdoff (1944). Basal leaves of many species have also been shown to be richer in manganese than apical ones by Jadin and Astruc (1913), Anderssen (1932), Liebig, Vanselow, and Chapman (1943), Bennett (1945), and Goodall (1949).

The Effect of Manganese on Photosynthesis

The study of the effect of manganese on photosynthesis is complicated by the effect of manganese on chlorophyll production. Many workers have noted the chlorotic condition of plants grown in the absence of manganese. McHargue (1926) is of the opinion that manganese is of equal importance to iron in the synthesis of chlorophyll. Friedrichsen (1944) found that increasing the manganese supply to chlorotic spinach plants from 2·5 μg./litre to 10μg./litre gave increased chlorophyll formation: maximum chlorophyll content was obtained when 50 μg./litre of manganese was supplied.

Decreased carbohydrate content in manganese-deficient plants has been noted by McHargue (1926), Bishop (1928), Miller (1933), Eltinge (1941), and Hunter (1942). Miller (1933) claimed that the great decrease in sugar content was too large to be accounted for by lowered chlorophyll content, and concluded that manganese plays a more fundamental role in sugar metabolism. This suggestion of Miller has been confirmed by some of the later work. Emerson and Lewis (1939) found an 85 per cent. increase in efficiency of photosynthesis in *Chlorella pyrenoidosa* on addition of a trace-element solution containing boron, zinc, copper, molybdenum, and manganese to the nutrient solution. Richter and Vassilieva (1941) sprayed dilute solutions of zinc sulphate, potassium permanganate, potassium iodide, and boric acid on to leaves of sunflower, bean, kok-saghyz, *Hydrangea*, and *Perilla* plants. In all cases an increase in photosynthesis resulted varying from 12 to 120 per cent. more than that of controls sprayed with distilled water. Manganese in the form of $KMnO_4$ raised assimilation rate by 12 per cent. in the sunflower. Doubling the concentration of $KMnO_4$ in the spray produced a 25 per cent. increase in assimilation. This increased rate lasted for several days, and only gradually decreased. No information is given as to the method of estimating carbon assimilation.

Gerretsen (1949) compared the carbon assimilation of detached oat leaves under high and low manganese levels. The assimilation rate was measured gasometrically by absorption in baryta. He found that the assimilation rate per unit area in the deficient leaves was greatly reduced. In one experiment the deficient leaves were chlorotic, but in the other experiments no perceptible difference in colour of leaves was noted, surprisingly, in view of the very low manganese content found in the leaves. Gerretsen therefore claimed that there is a direct effect of manganese on photosynthesis, independent of the indirect effect due to chlorosis.

Reuther and Burrows (1942) investigated the effect of the addition of manganese on photosynthesis of manganese-deficient tung-tree leaves. Pairs of shoots were selected showing various but equal degrees of 'frenching', and

one of each pair dipped into a solution containing 1 per cent. manganese sulphate and 0·5 per cent. calcium carbonate. Simultaneous measurements of photosynthesis were made on selected comparable leaves both from the tips and base of treated and control shoots. Carbon dioxide was measured by means of Heinicke cups attached to the leaves in the field.

A significant increase in photosynthesis was only found when top leaves were used in the two later periods. Basal leaves in all experiments showed no increase in photosynthesis. The important result emerges that the effect of the addition of manganese is relatively small and is found only in the youngest leaves.

Portsmouth (1949) determined the effect of manganese on carbon assimilation by means of a modified half-leaf method. Manganese-deficient potato plants were grown in a calcareous soil. The third or fourth leaf from the apex of a number of plants was selected and injected with a 0·025 per cent. manganese sulphate solution in such a manner that the level of manganese was raised only in the injected side of the leaf—the opposing leaflets acting as control series. Assimilation rates were measured 4 days after injection, and the increase in dry weight per unit area over a period of usually 6 hours was compared in the injected and control leaflets.

Portsmouth found that raising the manganese level caused a significant decrease in apparent assimilation. He concluded that this did not of necessity mean that there was a decrease in the real assimilation rate, and the possible effect of manganese in increasing translocation or respiration or both must not be overlooked.

Reviewing the results stated above, it will be seen that the evidence is conflicting. In Gerretsen's experiment, comparing manganese-deficient oat leaves with others containing high manganese, a very large effect on assimilation was obtained. In the experiments of Reuther and Burrows and of Portsmouth the method used was different, for here normal leaves were used and the manganese level suitably raised. Under these conditions no increase was obtained in assimilation rate in the basal leaves of tung by Reuther and Burrows, and only a slight increase in the apical leaves, while Portsmouth, with the potato, found an apparent decrease in assimilation rate after injection. In considering the problems of the effect of manganese on assimilation the mode of experimentation requires consideration.

1. *Assimilation rate of isolated rooted leaves*

Rooted leaves appear to be ideal material for the determination of the effect of manganese on carbon assimilation as by their isolation any effect of translocation from or to the leaf is avoided. An increase in the total weight of the rooted leaf throughout a period therefore provides a measure of its net assimilation.

Experimental method

Two assimilation experiments with isolated rooted leaves were carried out in 1951.

Detached leaves were rooted in culture solution as previously described. Some of these leaves were initially of high manganese content and were rooted and cultivated in high manganese nutrient solutions. Others, initially of low manganese content, were rooted and grown in low manganese solutions. A third series was obtained by rooting low manganese leaves in low manganese solution and transferring them to high manganese solutions. The three series are distinguished for convenience in presentation by low (L), transferred (LH), and high (H).

Random samples of rooted leaves were taken at the beginning of the experiment (t_0) and fresh weight, dry weight, and manganese content determined. Leaf areas were also obtained by a leaf print method.

A similar random sample taken at time (t_0) was allowed to grow on in the appropriate solution until time t_1, t_2, &c. (subscript number represents the duration of the experiment in weeks), when the plants were harvested.

Assimilation experiments

In expt. I, the high and low manganese rooted leaves were both grown in low manganese nutrient solutions for a 2 weeks' period. In expt. II high manganese leaves were grown in high manganese solution, low manganese leaves in low manganese solution, and a third series of low manganese leaves transferred to high manganese solution. This experiment was of 3 weeks' duration. The results obtained in expts. I and II are presented in Tables V and VI.

The data in Table V show the mean dry weight, mean leaf area, and mean manganese content of leaves and roots. It will be noted that with the high and low manganese rooted leaves the increase in leaf area was greater in expt. I than in expt. II; this may be related to the more advanced stage of development of the plants in the second experiment, or less favourable conditions of growth during that time.

The data in Table VI show the distribution of weight increases in the different tissues. It will be observed that in both experiments at the high manganese levels the greatest increase in dry weight has occurred in the leaflets, whereas at the low manganese level the increase in weight of leaflets is similar to that of the roots and is smaller in both cases when compared with the high manganese series.

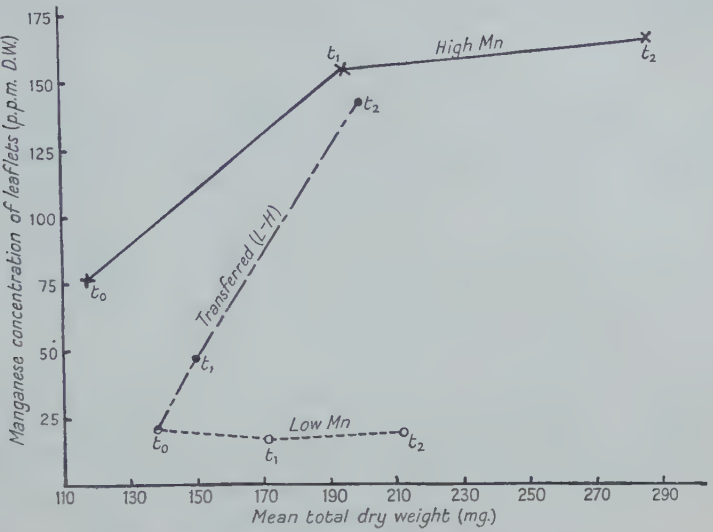
It will be seen that the leaves transferred from low to high manganese solutions behaved in a similar way to those grown in low manganese, and no apparent effect of manganese has occurred. In this respect, the results of this experiment confirm the findings of previous investigators in that high manganese content originally in the leaf leads to greater increases in dry weight; on the other hand, supply of manganese to deficient leaves after isolation has no effect on dry-weight increase. This absence of effect after transfer is, however, not due to the fact that manganese is not absorbed, as the data in Table V show. This result is most clearly seen in Text-figs. 1 and 2, from which it is apparent that in the laminae of leaves originally of high

TABLE V

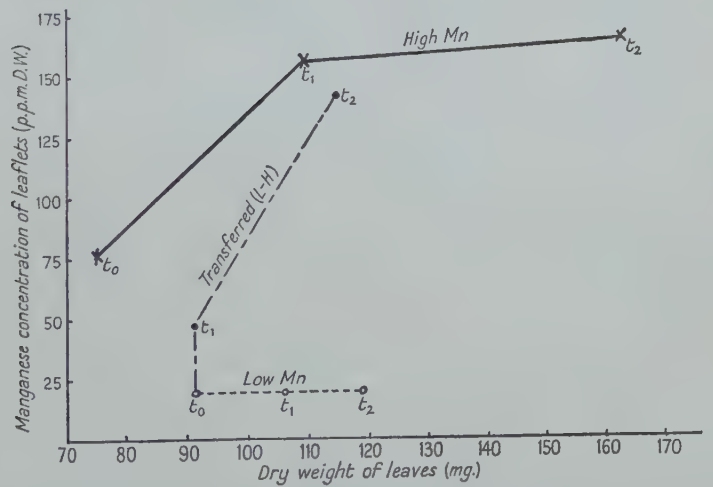
Mean Dry Weight of Rooted Leaves, Mean Leaf Area, and Mean Manganese Content of Leaves and Roots

Date 1951.	High Mn. D.W. (mg.).	High Mn. Leaf area (sq. cm.).	High Mn. Mn (p.p.m.) Leaves.	High Mn. Mn (p.p.m.) Roots.	Transferred (LH). D.W. (mg.).	Transferred (LH). Leaf area (sq. cm.).	Transferred (LH). Mn (p.p.m.) Leaves.	Transferred (LH). Mn (p.p.m.) Roots.	Low Mn. D.W. (mg.).	Low Mn. Leaf area (sq. cm.).	Low Mn. Mn (p.p.m.) Leaves.	Low Mn. Mn (p.p.m.) Roots.
Expt. I.	(In Mn-free solution.)								(In Mn-free solution.)			
t_0 22/5	174	26·4	47	—	—	—	—	—	135	20·6	17	—
	±13·5	±2·2	±4·0	—	—	—	—	—	±12·8	±1·7	±0·8	—
t_2 6/6	510	61·0	20	4	—	—	—	—	252	28·4	15	7
	±27·7	±3·5	±2·1	±0·8	—	—	—	—	±13·2	±1·7	±0·8	±0·6
	$N = 8$ in each sample for dry-weight, leaf-area, and manganese determination.											
Expt. II.	(In solution containing Mn.)				(Transferred to solution containing Mn.)				(In Mn-free solution.)			
t_0 18/7	117	23·8	77	937	138	29·2	20	14	138	29·2	20	14
	±5·4	±1·1	±8·8	±143	±11·6	±1·3	±1·5	±1·5	±11·6	±1·3	±1·5	±1·5
t_1 25/7	195	28·1	156	3068	149	23·3	48	670	171	25·5	18	25
	±14·6	±2·5	±37·3	±284	±10·7	±1·8	±1·1	±192·0	±13·3	±1·8	±0·5	±3·8
t_2 3/8	286	35·0	169	1906	199	32·9	142	4524	212	28·3	20	14
	±33·0	±4·4	±28·5	±327	±16·2	±2·9	±23·6	±627·0	±20·6	±2·3	±1·4	±0·3
	$N = 20$ in each sample for dry-weight and leaf-area determination.											
	$N = 10$,, ,, ,, ,, manganese estimation of leaves.											
	$N = 5$,, ,, ,, ,, manganese estimation of roots.											

manganese content the concentration of manganese continues to increase during the first week and then remains nearly constant; meanwhile the dry



TEXT-FIG. 1. Mean dry weight and manganese concentration of rooted leaves.



TEXT-FIG. 2. Mean dry weight and manganese concentration of leaflets of rooted leaves.

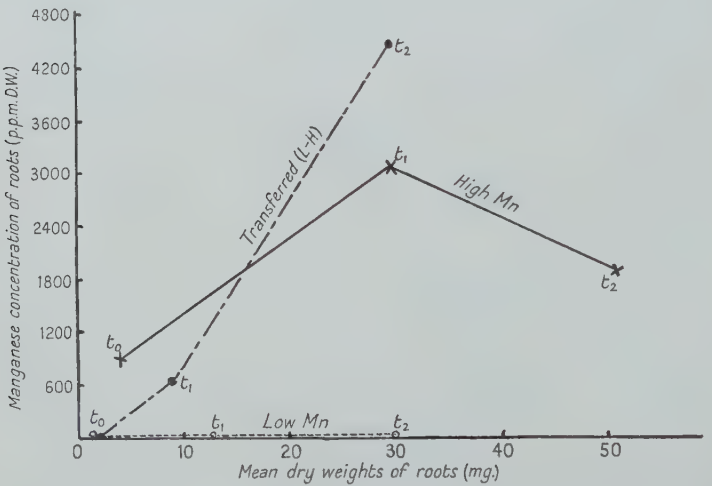
weight of the laminae steadily rises. These leaves were, however, supplied with manganese during this time. The low manganese leaves grown in a manganese solution showed an increase in weight and no change in manganese concentration. The transferred leaves, on the other hand, during the first week showed an increase in manganese concentration without any notable change in dry weight, and during the second week the manganese concentration

had reached almost the same level as the high manganese leaves, whereas the dry weight is similar to that of the low manganese leaves. In Text-fig. 3 a similar relation of manganese concentration and root growth is observed.

TABLE VI

Mean Dry Weight (mg.) of Lamina, Petiole, and Root System of Rooted Leaves

Date 1951.	High Mn (H). Leaflets.	Petioles.	Roots.	Transferred (LH). Leaflets.	Petioles.	Roots.	Low Mn (L). Leaflets.	Petioles.	Roots.
Expt. I.									
t_0 22/5	97	55	22	—	—	—	90	37	9·0
	±7·7	±5·9	±1·4	—	—	—	±7·3	±4·7	±1·8
t_2 6/6	286	112	111	—	—	—	138	53	61
	±18·6	±10·3	±4·9	—	—	—	±8·1	±2·6	±4·9
	$N = 8$ in each sample.								
Expt. II.									
t_0 18/7	74	38	4·0	91	45	2·0	91	45	2·0
	±3·5	±2·5	±1·1	±7·2	±4·7	±1·1	±7·2	±4·7	±1·1
t_1 25/7	108	56	30	90	51	9·0	105	53	13
	±7·5	±4·7	±6·5	±6·5	±4·0	±2·1	±7·4	±5·2	±2·5
t_2 3/8	161	74	51	114	55	30	118	65	30
	±19·9	±5·8	±10·4	±9·0	±3·7	±4·9	±10·7	±5·9	±5·9
	$N = 20$ in each sample.								



TEXT-FIG. 3. Mean dry weight and manganese concentration of roots of rooted leaves.

The very high manganese concentration found in the roots in high solution is probably spurious and due to precipitation of manganese on the root surfaces and is not a measure of the actual concentration in the tissues. Nevertheless manganese is absorbed after transfer as is shown by the difference of manganese contents of the laminae.

From the data collected by the usual method of growth analysis the net assimilation rate (increase mg. D.W. per sq. cm. per day) can be calculated. These results are shown in Table VII.

In both experiments the assimilation rate of the high manganese leaves was significantly greater than that of the other two treatments in spite of the higher respiration rate in the high manganese leaves which will be discussed later.

TABLE VII

Rooted Leaves. Assimilation Rate (mg.) per sq. cm. Leaf per Day

	High Mn.	LH Mn.	Low Mn.	Period weeks.	Range and significance.
Expt. I	0·542	—	0·343	2	t_0-t_2 $p < 0{\cdot}01$
Expt. II	0·420	0·064	0·175	1	t_0-t_1
	0·417	0·274	0·224	1	t_1-t_2 $p < 0{\cdot}001$
	0·411	0·151	0·186	2	t_0-t_2
Mean	0·448	0·163	0·186	—	—

Sampling times t_0 = initial; t_1 = after 1 week; t_2 = after 2 weeks.

A rough estimate of the chlorophyll content of the rooted leaves was made at each sampling period on five leaves in each treatment. The leaflets from each rooted leaf were extracted in 30 ml. boiling alcohol, the solution diluted to one-tenth, and the colour estimated colorimetrically. Table VIII gives a comparison of chlorophyll values per 1 g. F.W. of leaflets.

TABLE VIII

Chlorophyll per 1 g. F.W. Leaflets

Expt. II.	High Mn.	LH Mn.	Low Mn.
t_0	10·5 ±0·8	9·6 ±1·1	9·6 ±1·1
t_1	10·1 ±0·5	9·4 ±0·4	9·6 ±0·9
t_2	8·7 ±0·7	9·1 ±0·6	7·6 ±0·4

Five replicates in each sample.

No significant difference was found between any of the treatments. Thus manganese has shown a strong positive effect on assimilation which is independent of chlorophyll content.

Manganese and translocation

The effect of manganese on the distribution of assimilate to the various organs will be shown by the relative changes in weight of the parts. The percentage partition of the increment in dry weight over the experimental period to the leaf, petiole, and root can therefore be used as a measure of the effect of manganese on translocation (Table IX), since material for the growth of roots must be derived from laminae.

It will be observed that in the high manganese treatments a greater percentage of dry matter has been retained by the leaves. This difference just fails to reach significance ($p < 0{\cdot}1$–$0{\cdot}05$).

This distribution of dry matter between root and lamina does not unequivocably establish the fact that manganese has no effect on translocation,

since the increment in root weight will depend not only on supply of assimilates from the leaf but on the number of active meristems in the roots, and it has already been suggested earlier that under conditions of low manganese lateral root formation is reduced.

From the experiments therefore there is no evidence of any positive effect of manganese on translocation.

TABLE IX

Percentage Distribution of Increment Dry Matter in Rooted Leaves

	High Mn.			LH Mn.			Low Mn.		
	Leaflets.	Petioles.	Roots.	Leaflets.	Petioles.	Roots.	Leaflets.	Petioles.	Roots.
Expt. I.									
t_0–t_1	56·2	17·0	26·3	—	—	—	41·0	13·7	44·5
Expt. II.									
t_0–t_1	43·5	23·0	33·3	−9	54·5	57·2	42·4	24·1	33·3
t_0–t_2	51·5	21·3	27·8	37·8	16·4	45·8	36·5	27·0	37·8
t_1–t_2	58·3	19·8	23·1	48·0	8·0	42·0	31·7	29·3	41·5
Mean	52·4	20·3	27·6	25·6	26·3	48·3	37·9	23·5	39·3

2. *Assimilation experiments with rooted shoots*

An experiment similar to those already described with rooted leaves was carried out on rooted shoots. On May 25, 1951, sixty small shoots were detached from tubers, placed in manganese-free sand, and after rooting transferred on June 9 to 600-c.c. beakers containing low manganese nutrient solutions.

On June 12 three representative samples of ten plants each were chosen. At this time no symptoms of manganese deficiency were present on the plants. On one sample of ten, fresh weights and dry weights, leaf areas, and manganese contents of leaflets and roots were determined. The second sample was allowed to grow on in low manganese solution, and the third transferred to a high manganese solution.

These two series were grown on until June 20, when first signs of manganese deficiency became apparent in the low manganese series—a slight fine spotting on apical leaves of one plant—there was no apparent difference in greenness of the two series. The plants were sampled as before and chlorophyll determinations made.

Table X gives the mean dry weights, mean leaf areas, and the mean manganese content of leaflets and roots. Table XI gives the mean dry weight of leaves, stems, and roots.

The figures in Table X show that the difference in percentage increase in dry weight and leaf area as between high and low manganese was 29 per cent. and 63 per cent. respectively. This greater percentage increase in dry weight is shown in leaves, stems, and roots.

The high manganese plants also show a significantly greater net assimilation rate ($P < 0{\cdot}05$), the values for high and low manganese being 0·424 mg.

and 0·300 mg. per sq. cm. per day respectively, which agrees with the values obtained with the isolated rooted leaves.

Chlorophyll estimations were made on the leaves of three plants of each series, using the method previously described, the values for high and low manganese being 14·8 and 15·6 respectively.

No significant difference in chlorophyll content was found; thus a difference in net assimilation rate was found which was not related to chlorophyll content.

The percentage distribution of increment of dry weight during the experimental period to the leaves, stems, and roots is shown below.

High Mn.			Low Mn.		
Leaves.	Stems.	Roots.	Leaves.	Stems.	Roots.
56·6	25·4	17·5	55·5	25·7	18·1

The result of this experiment with rooted shoots has confirmed the results in general obtained with the rooted leaves, viz. that manganese has a direct effect on carbon assimilation independent of chlorophyll concentration, and has no marked effect on translocation.

TABLE X

Behaviour of Rooted Shoots grown in Low Manganese and afterwards transferred to High Manganese Solution. Mean Dry Weight, Mean Leaf Area, and Manganese Content

	Transferred to high Mn solution.				Low Mn.			
			Mn p.p.m.				Mn p.p.m.	
Date 1951.	D.W. (mg.).	Leaf area (sq. cm.).	Leaves.	Root.	D.W. (mg.).	Leaf area (sq. cm.).	Leaves.	Root.
t_0 13/6	252 ±30·7	31·2 ±3·5	25 ±1·2	18 ±1·8	252 ±30·7	31·2 ±3·5	25 ±1·2	18 ±1·8
t_1 20/6	469 ±37·6	104·7 ±8·1	140 ±10·3	1118 ±319·0 ($N = 5$)	396 ±29·6	85·1 ±6·4	19 ±2·8	14 ±0·7 ($N = 5$)

Ten replicates in each example.

TABLE XI

Mean Dry Weight Leaves, Stems, and Roots

	Transferred to high Mn solution.			Low Mn.		
Date 1951.	Leaves.	Stems.	Roots.	Leaves.	Stems.	Roots.
t_0 13/6	73 ±10·1	156 ±23·1	24 ±2·8	73 ±10·1	156 ±23·1	24 ±2·8
t_1 20/6	196 ±20·5	211 ±21·0	62 ±6·3	153 ±13·8	192 ±17·0	50 ±5·9

Ten replicates in each sample.

The Effects of Manganese on Respiration

There is some evidence that manganese promotes oxidation-reduction reactions in the plant.

Bertrand (1897) added manganese to a relatively manganese-free enzyme extract from lucerne (*Medicago sativa* Linn.) in hydroquinone solution and found an increase in oxygen uptake.

Gerretsen (1950) claimed that in a crude suspension of chloroplasts the addition of manganese produced a temporary increase in rate of oxygen uptake in the light. Similar catalytic effects of manganese are well known in non-living systems (e.g. the 'drying' of linseed oil) and the results obtained with a crude suspension of plant material are open to a number of possible interpretations.

Lündegardh (1939) using intact roots of wheat plants showed that oxygen uptake was increased by 76 per cent. on the addition of 0·00005 M. $MnCl_2$ solution to an oxygenated 0·001 M. KCl solution. In a similar experiment the addition of manganese to a 0·004 M. KNO_3 solution produced a 47 per cent. increase in oxygen absorption. He claimed that manganese therefore was concerned in oxidation processes involved in respiration. This conclusion was also reached by Arnon (1937) and Wallace (1951).

Gerretsen (1949) determined the carbon dioxide produced by respiration of normal and deficient oat leaves. He found a 30 per cent. decrease in respiration in the deficient leaves. It is evident that lack of manganese is associated with a decrease in respiration, but no data as regards condition of material, manganese content, or significance of results are given.

Gerretsen concluded that the difference in respiration rate may be an indirect one, increased carbon assimilation in the high manganese series resulting in more carbohydrates being available for respiration.

Respiration Experiments

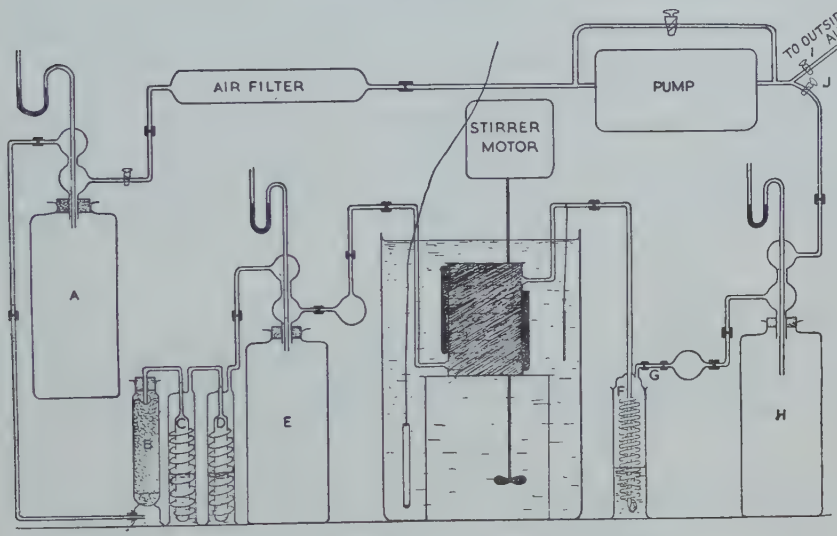
Respiration apparatus

The technical details of the respiration apparatus used are shown in Text-fig. 4. Essentially the apparatus consists of eleven respiration chambers arranged in parallel and immersed in a water-bath thermostatically controlled within $\pm 0{\cdot}02°$ C. by means of an aquarium heater, stirrer, mercury toluene regulator, and 'Sun-Vic' relay.

The respiration chambers, of a size suitable for rooted potato leaves, were made of ordinary horticultural glass, the internal dimensions being $20 \times 11 \times 2$ cm. The glass plates and strips were coated at their edges with 'Araldite' powder, held by a metal jig and heated in a hot-air oven for 1 hour at 180° C. As a precautionary measure a coating of liquid 'Araldite' was applied when the container had cooled. Connecting tubes were cemented in with liquid 'Araldite' to holes previously drilled at the top of one side and bottom of the other side of each chamber. These joints were not altogether satisfactory and

had to be renewed at intervals. Stepped glass lids composed of a large outer and smaller inner pieces were built up; these when luted with vaseline gave a satisfactory joint. The chambers were weighted by means of test-tubes filled with lead shot attached to each side. To exclude light the chambers and lids were coated externally with bitumen.

Air was circulated in a 'push-pull' system by means of an Edwards vacuum-pressure pump. Before passing through the chambers the air was freed from traces of oil vapour by an Edwards oil baffle followed by a 6-inch column of



TEXT-FIG. 4. Diagram of respiration apparatus.

closely packed cotton-wool. The air was then passed into a reservoir (A) with a manometer attached. On leaving the reservoir it was freed from CO_2 by passage through a 'Carbosorb' soda-lime tower (B), scrubbed through 10 per cent. potassium hydroxide (C), and finally through $n/10$ barium hydroxide solution (D). The air was next circulated into a reservoir (E) with manometer attached and thence to the respiration chambers. After passage over the respiring material, air was freed from CO_2 by absorption in barium hydroxide solution in a series of bubblers (F). Each absorption vessel consisted of a glass spiral which fitted loosely into an upward tube drawn to a fine jet; in this way the air stream was broken up into a series of fine bubbles in the spiral, ensuring good absorption of the carbon dioxide in the barium hydroxide solution. The rate of flow of the air stream was stabilized in all the vessels by means of short capillary tubes (G). The air was then passed into a third reservoir (H) with manometer and returned to the circulatory system. A variable 'by-pass' to the pump was found necessary for controlling the flow.

By suitable adjustment of the apparatus it was possible to keep the pressure in the respiration chambers within 1 per cent. of atmospheric, thus minimizing any tendency to leakage, and making it possible to attach and detach

absorption vessels without in any way disturbing the rate of flow in the others.

At the beginning of each respiration experiment the apparatus was flushed with air drawn from outside the laboratory. Stopcock (J) was then opened and stopcock (I) closed, thus causing the air to circulate over the respiring material. In all experiments two chambers were left empty and the titration of the absorption vessels connected with these were used as blanks.

Normally 25 c.c. of approximately $n/10$ barium hydroxide were pipetted into the absorption vessels from an automatic pipette, and after absorption of CO_2 were titrated with $n/10$ HCl, using phenolphthalein as indicator. A closed system with mechanized stirring was used. Highly consistent respiration results were obtained.

Respiration rates were calculated as mg. CO_2/100 g. F.W. per hour. All respirations were carried out at 25° C. for a period of 4 hours. The experimental material was allowed to remain in the chambers with air flow at normal rate (approx. 50 c.c. per minute) in each chamber for 30 minutes before the absorption vessels were connected.

Experiments with rooted leaves

Respiration experiments with high and low manganese rooted leaves were carried out. Particular attention was paid to the position of the leaf on the plant before rooting and leaves of the same position were compared after

TABLE XII

Respiration of Isolated Rooted Leaves (25° C.) (mg. CO_2/100 g. F.W. per Hour)

Date 1951.	Position from apex.	High.	Mn p.p.m.	Low.	Mn p.p.m.
21/5	4th	38·5	66	39·8	16
	,,	35·0	52	31·7	18
	,,	45·8	47	31·3	21
	,,	35·3	58	41·3	15
29/5	6th	35·3	48	35·6	27
	,,	38·5	46	30·5	21
	,,	32·8	61	34·3	25
	,,	33·1	33	30·5	14
	,,	—	—	41·0	15
	,,	—	—	31·4	20
	,,	—	—	37·7	14
	,,	—	—	34·7	16
	,,	—	—	38·3	19
	,,	—	—	21·8	24
	,,	—	—	34·3	13
	,,	—	—	29·5	24
17/7	2–8th	50·9	76	30·3	15
	,,	37·7	71	31·5	20
	,,	43·4	64	35·5	20
	,,	38·6	102	—	—
Mean		38·7	61·0	33·8	19·0

rooting (Table XII), preliminary experiments having shown that with unrooted detached leaves age or position of leaf on the plant was an important factor in determining the respiration rate.

A significant increase in respiration ($P = 0{\cdot}02$–$0{\cdot}01$) of the high manganese

TABLE XIII

Effect on Respiration of Rooted Leaves of Transfer from Low to High Manganese Solution

Date. July 9, 1951	Before transference to manganese solution. Respiration (mg. CO_2/100 g. F.W./hr.).	Mn p.p.m. D.W.
	39·9	16
	51·9	23
	48·5	26
	38·5	24
	45·9	20
	54·8	21
	55·2	22
	47·8	21
Mean	47·8	21·5

	Mn+ solution.		Mn− solution.	
Period.	Respiration.	Mn.	Respiration.	Mn.
20 hrs.	32·9	20·0	38·5	19
	38·6	42	33·5	21
	34·5	21	36·3	20
	33·5	26	32·6	18
Mean	34·9	27·3	35·2	19·7
40 hrs.	36·0	26	23·8	23
	30·2	32	28·7	27
	28·5	27	21·5	25
	27·0	23	—	—
Mean	30·4	27·2	24·6	24·8
80 hrs.	31·4	18	33·3	14
	25·9	96	16·7	30
	29·0	17	30·5	21
	32·1	20	24·7	17
Mean	29·8	37·6	26·3	20·3

plants was found, although the total effect was small. There is no evidence from the above data that manganese has stimulated respiration directly. The effect noted may be due to increased carbohydrate content in the high manganese rooted leaves.

To test this hypothesis, the respiration rate of low manganese rooted leaves that had been transferred for different periods of time to high manganese nutrient solution was determined, the leaves remaining in the low manganese solution serving as controls. In this manner it was hoped that any direct stimulation of manganese on respiration would be observed.

On July 9, 1951, twenty-four low manganese rooted leaves were weighed and grouped by weight into eight similar batches of three leaves each. Respiration of each group was determined over a 4-hour period and samples taken for manganese analysis. Immediately after removal from the respiration apparatus, four groups of leaves were transferred to a high manganese solution. Res-

TABLE XIV

Before Transference to Manganese Solution

Date.	Respiration (mg. CO_2/100 g. F.W./hr.).	Manganese. p.p.m. D.W.
July 23, 1951	35·3	28
	40·9	12
	37·8	19
	39·0	14
	37·7	15
	42·6	18
	34·1	35
	33·5	21
Mean	37·6	Mean 20

	20 hrs. in Mn+ solution.		20 hrs. in Mn− solution.	
	Resp.	Mn.	Resp.	Mn.
	34·1	16	30·9	31
	31·7	19	36·3	14
	34·1	17	38·3	20
	29·3	16	30·9	—
Mean	32·3	17	34·1	22
	40 hrs. in Mn+ solution.		40 hrs. in Mn− solution.	
	33·5	11	31·7	17
	34·1	15	32·9	31
	39·4	14	33·3	—
	26·6	13	30·1	13
Mean	33·5	14	32·0	20
	80 hrs. in Mn+ solution.		80 hrs. in Mn− solution.	
	22·7	86	20·8	17
	22·9	23	32·1	20
	23·7	20	30·7	27
	21·7	19	18·6	—
Mean	22·7	37	25·5	21

piration rates of these groups and of the control series were then determined after 20 hours, 40 hours, and 80 hours, leaflets being taken at these times for manganese analyses. Table XIII gives the respiration results and manganese concentrations at these times.

The same experiment was repeated on July 23, 1951, using fresh material. Thirty-two low manganese rooted leaves were selected and a similar technique used. Table XIV gives the respiration results and manganese concentrations.

In view of the fact that the manganese content of the leaflets of low manganese rooted leaves transferred to high manganese solution has not increased,

even at the end of an 80-hour period, except for one leaflet in each series, any effect of added manganese in respiration of leaflets could not be studied with this material. The manganese content of the roots of these leaves was not determined as such determinations are not satisfactory owing to the fact that it appears probable that manganese is precipitated on the surface of the roots. The important consideration is whether manganese reaches the lamina. An experiment carried out after 7 days' transfer to high manganese solution showed a mean increase of manganese from $20 \pm 1{\cdot}5$ to $48 \pm 11{\cdot}1$ ($N = 10$) p.p.m. D.W., and that this increase was correlated with the size of the root system ($r = +0{\cdot}873$; $d.f = 17$; $p < 0{\cdot}001$).

Rooted shoots

Small sprouted tuber pieces were planted in manganese-free sand on May 25, 1951. Rooting began within 14 days. On June 9 the rooted sprouts

TABLE XV

Respiration Rates of Rooted Shoots (T. 25° C.) in Low Mn solution before transfer to high Mn solution

Plant group.	F.W. (g.).	mg. CO_2/100 g. F.W./hr.
1	10·6	24·5
2	13·9	24·3
3	13·3	22·3
4	10·5	27·8
5	11·7	23·5
6	13·9	24·8
7	13·3	25·9
8	11·7	24·5
	Mean	24·3

were detached from the tubers and planted in 600-c.c. Pyrex beakers containing a manganese-deficient nutrient solution and given the cultural treatment already described. In this manner small plants of low manganese content, some showing typical deficiency symptoms, were produced. The shoots were weighed singly and grouped into lots of approximately 10 g. F.W. each, and the respiration rates determined for the usual 4-hour period on June 11, 1951. (Table XV.)

At the end of the respiration period eighteen plants were selected at random and nine of them transferred to a high manganese solution. After a period of 24 hours the respiration rates of the transferred plants were compared with those of the controls. (Table XVI.)

On September 20, 1951, a similar experiment was repeated on a larger scale.

Detached shoots, all showing manganese-deficiency symptoms on their apical leaves, were grouped at random into eight similar batches and respiration rates determined. Manganese analyses were done on comparable leaflets on four groups (Table XVII.)

TABLE XVI

Respiration Rates (mg. CO_2/100 g. F.W./hr.) and Manganese Content (p.p.m.) after 24-hour period

	Transferred plants.		Controls.	
	Respiration.	Mn.	Respiration.	Mn.
	22·7	35	16·3	28
	20·0	44	20·5	33
	20·4	45	21·9	22
	24·5	42	18·6	32
	—	—	23·3	27
Mean	21·9	41·0	20·1	28·0

TABLE XVII

Respiration Rates (mg. CO_2/100 g. F.W./hr.) and Manganese Content (p.p.m.)

Plant group.	Respiration rates.	Manganese content.
1	35·3	11
2	32·7	—
3	30·1	18
4	29·0	—
5	30·5	8
6	32·1	—
7	24·6	10
8	30·5	—
Mean	30·6	12

TABLE XVIII

Respiration Rates (mg. CO_2/100 g. F.W./hr.) and Manganese Content (p.p.m.) of Rooted Shoots, after Transfer to High Mn at Stated Times

Transferred plants.		Mn.	Controls.	Mn.
18 hrs. in Mn solution.				
	26·7	14	28·9	11
	27·7	36	36·5	13
	33·4	16	30·5	9
	32·1	17	35·3	9
Mean	30·0	21	32·8	11
6 days in Mn solution.				
	34·1	51	38·0	—
	34·1	34	32·0	4
	38·0	—	34·2	5
	40·7	70	34·4	8
Mean	36·7	52	34·6	6

At the end of the respiration measurement approximately half of the material was transferred to a high manganese solution, the remainder being grown on in the deficient solution.

The respiration rates of the controls and transferred plants were compared after 18 hours and again after 6 days. (Table XVIII.) No difference in appearance of the plants was noted after 18 hours, both lots of plants showing chlorosis and necrotic areas on the apical leaves. After 6 days the young leaves of plants transferred to high manganese solution had become green, although spots were still present on some of the lower leaves. The deficient plants were by this time very chlorotic and spotted.

It will be observed that in contrast to the rooted leaves the rooted shoots have taken up manganese after 18 hours in a high manganese solution, and that after 6 days the manganese concentration had risen considerably. No significant stimulation of respiration rate by manganese was, however, found at either period.

It may be concluded that manganese has had no direct effect on respiration of this material.

Discussion

The main object of the experiments previously described was to determine whether: (1) manganese level had a direct effect on assimilation rate; (2) translocation rate was influenced by manganese content; (3) manganese had an effect on respiration.

The evidence for the effect of manganese on assimilation as stated earlier in this paper is conflicting, for while Gerretsen produced evidence to show an increase of assimilation, Portsmouth from his experiments, using a modified half-leaf method, concluded that manganese had no direct effect on assimilation, and that net assimilation rate was actually decreased by injected manganese. To account for this result he suggested the following possibilities: (*a*) manganese increased translocation from the leaf; (*b*) manganese increased respiration rate.

In the work here presented there is good evidence to show that the net assimilation rate of leaves detached from high manganese plants and subsequently rooted was higher than that from low manganese plants. Such a comparison is valid only if the low manganese leaves had not reached the stage of deficiency at which necrotic areas are present, and in most of the experiments reported no symptoms of this kind were present. One of the symptoms found in deficient leaves is the appearance of chlorosis, and in view of this chlorophyll estimations were made on the normal and deficient leaves. No consistent differences were found. The discrepancy between results here recorded and those of Portsmouth require consideration. Portsmouth in his experiments used plants grown in manganese-deficient soil from a drained lake on which plants grown *in situ* show characteristic symptoms of manganese deficiency. The plants were injected at the time at which no such symptoms had yet appeared. The analyses for manganese on these leaves

showed a rather low value (17 p.p.m. D.W.), but the method for the manganese extraction used at that time is now known to give low values, so that it is by no means certain that the leaves were below the critical manganese level for normal functioning. The experiments shown in Table V, carried out with rooted leaves low in manganese and transferred to a high manganese solution, show that although the manganese content of the leaf can be considerably increased there is no increase in the assimilation rate, and in Portsmouth's experiment the increase in manganese by injection is essentially similar; the inconsistency of the results may therefore be more apparent than real.

It would appear possible that the effect of manganese on the assimilation apparatus occurs during the development of the leaf and once the chloroplast system has developed to its full extent any deficiency in manganese occurring during the development of the chloroplast cannot subsequently be made good. It must, however, be observed that the capacity for formation of chlorophyll in manganese-deficient leaves is not lost as considerable greening can occur after injection. This is only true if the deficiency has not advanced beyond a certain stage, for under these conditions after manganese addition the leaf dies.

The factors leading to chlorophyll production, of which manganese supply is one, may, on the other hand, be quite different from those concerned with maintenance of normal assimilation, and the data cited show no direct correlation between chlorophyll and assimilation over a wide range of manganese content, for with similar chlorophyll contents assimilation rate may vary considerably. From the design of Portsmouth's experiment comparisons of assimilation rates were made between injected and non-injected leaflets of leaves already low in manganese. The control in this case was therefore also low in manganese. This differs essentially from the experiment with rooted leaves in which high manganese leaves were used as controls, and therefore if the suggestion put forward is correct, viz. that once deficient in manganese mature leaves cannot be made to assimilate at the normal rate by supplying manganese, it follows that the results obtained with rooted leaves are not directly comparable to those obtained by injection. The only valid comparisons that can be made with Portsmouth's experiment are those in which rooted leaves were transferred from a manganese-free solution to a normal solution, in which case no increase in assimilation resulted in spite of the fact that the manganese content had risen considerably. As already stated, Portsmouth produced evidence to show that a decrease in apparent assimilation occurred after injection, and to account for this he suggested that either respiration losses were increased or that translocation rate was increased by high manganese. In the work here reported both these suggestions have been examined.

The large body of respiration data presented indicates that the effect of manganese on respiration is comparatively small. Higher respiration was consistently associated with higher manganese content (average figures being a 15 per cent. increase) and therefore insufficient to account for Portsmouth's results.

The data for the rooted leaves make possible an estimation of the effect of manganese on translocation, since the root system is entirely adventitious and its growth depends on the supply of materials from the laminae; the final size of the root system will depend on translocation from the laminae. The distribution of the increment in weight during the experiment between the lamina, petiole, and root therefore is a measure of translocation. There is no evidence that translocation has been increased by supplying manganese, though, as pointed out earlier, the size of the root system is not entirely determined by supply, as the branching of the primary root is also of importance. It appears that in low manganese solution branching is much less. Whether this is due to limitation by carbohydrate supply or to some specific effect of manganese in production of laterals cannot be determined from these experiments.

Summary

1. Potatoes were grown in sand and water cultures to provide material for experiments on the effect of manganese on carbon assimilation, respiration, and translocation.

2. Methods of propagation by means of (*a*) tubers, (*b*) detached sprouts, (*c*) leaf-bud cuttings, (*d*) isolated rooted leaves are described and details of cultivation given.

3. Manganese was estimated by means of a modified 'tetrabase' method which is described.

4. Analyses showed a close agreement in manganese concentration between the leaflets on any one leaf. The position of the leaf on the plant has a marked effect on manganese concentration, the basal leaves having more manganese than the apical ones.

5. Isolated rooted leaves never attained the size of leaves attached to the plant.

6. Rooting of low manganese leaves was found to be difficult. Their growth both in lamina and root was much less than that of high manganese leaves. In no case did the manganese-deficient rooted leaf develop characteristic deficiency symptoms, although the manganese level had fallen below that of leaves which show symptoms when attached to the plant.

7. A highly significant difference in net assimilation rate was found between leaves which had a high or low manganese content *at the time of rooting*. The addition of manganese after rooting to low manganese leaves did not, however, cause an increase in assimilation rate. Possible reasons for the failure of the 'deficient' rooted leaves to respond to manganese are discussed.

8. No evidence for any effect of manganese on translocation of assimilate from the leaf to root was found.

9. Parallel experiments with detached sprouts confirmed the positive effect of manganese on carbon assimilation, and absence of an effect on translocation.

10. The effect of manganese on respiration of rooted leaves and detached

sprouts at different manganese levels was studied. It was found that high manganese leaves have a significantly higher respiration rate (15 per cent.). The addition of manganese over a period of 1 week to 'deficient' rooted leaves and detached sprouts did not increase respiration, thus there is no evidence for any direct effect of manganese on respiration rate.

Acknowledgements

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EXPLANATION OF PLATE XV

Effects of Manganese and α-Naphthalene Acetic Acid on the Rooting of Isolated Leaves.

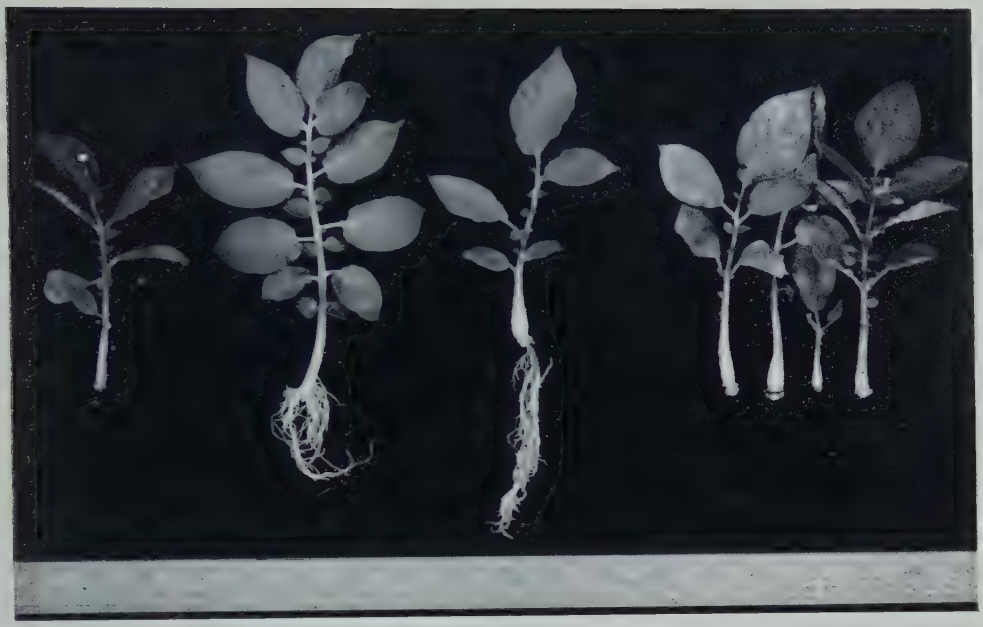

PLATE *a*. Cuttings treated with α-naphthalene acetic acid (2 p.p.m.). All two months after treatment.

Left to right:—1. No rooting; 2. Rooted cutting; 3. Rooted cutting with swollen base; 4. Four cuttings showing swollen bases and callus, but no rooting.

PLATE *b*. Typical healthy and manganese-deficient leaves six weeks after rooting.

The Effect of Molybdenum upon the Nitrogen Metabolism of *Anabaena cylindrica*

I. A Study of the Molybdenum Requirement for Nitrogen Fixation and for Nitrate and Ammonia Assimilation

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With three Figures in the Text

Abstract

The effect of molybdenum concentration upon the growth of *Anabaena cylindrica* is studied. Within the range of molybdenum concentration that it was found possible to achieve by the methods described, the element does not affect growth on ammonium chloride, but it is required for healthy growth on nitrate or gaseous nitrogen, the optimal concentrations being about 0·075 and 0·20 p.p.m., respectively. Molybdenum appears to be unnecessary for nitrate uptake.

Introduction

IT was reported by Bortels in 1940, and confirmed by Fogg in 1949, that molybdenum has a catalytic effect on nitrogen fixation by pure cultures of *Anabaena cylindrica*, a member of the Nostocaceae. The following report is based on a more detailed study of this effect, and of the molybdenum requirement of nitrate and ammonia assimilating cultures of the same organism. Pure cultures of *A. cylindrica* were obtained from Dr. Fogg, to whom I am also indebted for details of culture media and methods, and of the results of his experiments on the nitrogen metabolism of the alga.

Methods and Materials

Special precautions were necessary to ensure that the media used in this study were free of molybdenum impurities. The metal was removed from the macronutrients—potassium dihydrogen phosphate, magnesium sulphate, calcium chloride, and, if used, potassium nitrate or ammonium chloride—by co-precipitation of the metal ion with iron and 8-hydroxyquinoline in the presence of acetic acid (Nicholas and Fielding, 1950). This method is unsuitable for purifying micronutrients since it is not specific for molybdenum; 8-hydroxyquinoline combines readily with iron, manganese, zinc, and copper. The trace-element salts, manganese sulphate, boric acid, zinc sulphate, and copper sulphate, were purified by triple crystallization from pyrex-distilled water, and ferric chloride by recrystallization from redistilled alcohol.

The medium (for composition see Fogg, 1949) was prepared with pyrex-distilled water, and pyrex glassware was used throughout. The concentrations of potassium nitrate and ammonium chloride, when these salts were used in the medium, were 2·0 g. per litre and 1·0 g. per litre, respectively. When a

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sample of the medium was concentrated two hundredfold and tested with 4-methyl-1:2 dimercaptobenzene (which reacts with molybdenum to give a green-coloured derivative) it gave negative results, indicating that the medium could not have contained more than 0·0005 p.p.m. of molybdenum. The complete absence of molybdenum could not be confirmed; there is no colorimetric method known to the author, sensitive at these low concentration levels, and emission spectra, even in the ultra-violet region, would not reveal traces of molybdenum in solutions containing comparatively high concentrations of other metal ions (Mitchell, 1948).

Experiments with 'air flow' type of apparatus

A pure culture of *Anabaena* was subcultured three times in a molybdenum-free medium containing ammonium chloride as the nitrogen source, before being used in the first experiment. The cultures could not be left in the same ammonium chloride solution until maximum growth had been attained (3 weeks: Fogg, 1944) because of the deleterious effect on the alga of the increasing acidity of the medium. This resulted from the absorption of the ammonium ion and could not be balanced by increased buffering as *Anabaena* is also sensitive to phosphate concentration and is adversely affected by concentrations as low as M/15. The alga was, therefore, subcultured every sixth day.

During the experiments the alga was grown in 200-ml. pyrex Drechsel bottles, each containing 120 ml. of medium, aerated with a carbon dioxide-enriched air supply (0·5 per cent. CO_2) at the rate of 4 litres per hour per bottle. The air and carbon dioxide were freed from oxides of nitrogen by passing them through a 1 per cent. solution of sodium bicarbonate, followed by a 25 per cent. solution of sulphuric acid. The Drechsel bottles were immersed in water maintained at 24·5–25·5° C. and the alga was illuminated by a submerged 250-Watt tungsten lamp giving a light intensity of 200 foot-candles. The same amount of alga, determined by measuring the total filament length (Fogg, 1944), was inoculated into each flask in each experiment. The only variables were molybdenum concentration and the nitrogen source. Six molybdenum concentrations ranging from zero to 0·2 p.p.m. were used, in duplicate, in each experiment. The molybdenum was supplied as sodium molybdate.

After a growth period of 18 days the alga was harvested and samples from each bottle taken for dry weight and total nitrogen determinations. Total nitrogen was estimated by the standard micro-Kjeldahl method. A selenium catalyst was used in the digestion process, and digestion was continued for 8 hours after the solution had cleared.

Experiments were carried out with gaseous nitrogen as the only nitrogen source, and with either potassium nitrate or ammonium chloride as the source of nitrogen. In the case of ammonium chloride assimilation the cells were harvested, for reasons discussed above, after 14 days of growth.

The results are summarized in Table I.

Discussion of Results

It is apparent from Table I that:

1. Within this experimental range of molybdenum concentration, molybdenum does not affect the amount of growth during ammonia assimilation. The lower yield, as compared with that for nitrate and nitrogen assimilation, is probably due more to the adverse effects of the pH, which had fallen to 5·9 at the end of the growing period, than to the shorter growth period.

2. Increased molybdenum does lead to an increase in growth in the other two cases—of 150 per cent. above that without it for nitrate assimilating cultures, and of 200 per cent. for nitrogen-fixing cultures.

Table I

The Effect of Molybdenum Concentration on the Growth of Nitrogen-fixing, Nitrate assimilating, and Ammonium assimilating Cultures of A. cylindrica

Growth is measured in terms of dry weight and total nitrogen. In the nitrogen-fixing series each point is the mean of duplicate samples from three individual experiments ± the standard error of the mean. In the nitrate and ammonium assimilating series each point is the mean of duplicate samples from two experiments ± the standard error of the mean.

Molybdenum concentration (p.p.m.).	Dry weight (mg.)			Total nitrogen (mg.)	
	N_2.	KNO_3.	NH_4Cl.	N_2.	KNO_3.
0	67± 9·8	103±14·0	163± 4·2	4·74±0·79	7·92±1·35
0·02	95±13·8	184± 6·5	165± 2·8	7·07±1·68	14·58±0·55
0·05	128± 7·3	207± 8·5	153± 4·9	9·53±1·62	14·46±0·36
0·075	154±10·6	242± 8·0	150±12·7	11·49±1·54	18·11±0·36
0·10	171±16·7	241± 6·5	175± 2·1	13·23±2·52	17·83±0·57
0·20	201±10·6	247± 1·5	163± 6·3	16·49±1·62	18·63±0·08

3. Maximal growth in the presence of nitrate is reached at a molybdenum concentration of 0·075–0·1 p.p.m. whereas maximal growth during nitrogen fixation requires just over 0·20 p.p.m. The reason for this difference in optimal requirement has yet to be elucidated.

4. There is still appreciable growth in the media to which no molybdenum has been added. It was noticed that there was little visible increase in flasks without molybdenum during the first few days after inoculation, but that the cultures then began to grow quite steadily, although unhealthily. This suggested that:

(*a*) A small amount of nitrogen might be fixed, or assimilated by some route other than that involving molybdenum and that this process might require a period of adaptation; or,

(*b*) The medium might not have been completely deficient in molybdenum. Since its effect at concentration levels as low as 0·002 p.p.m. is still appreciable, concentrations of just less than 0·0005 p.p.m. might be sufficient to sustain a limited amount of growth.

There were also possible sources of contamination, namely, the pyrex culture bottles, the cotton-wool present in the bacterial-filter tubes attached to the Drechsel bottles and in the plugs used in autoclaving them, and the rubber tubing of the air-feed lines. The first two sources were checked and eliminated as sources of contamination, but the third was still open to doubt. Water vapour, condensing in the tubing, might dissolve out small amounts of molybdenum impurity which would then, should the condensation droplets fall into the medium, be available to the alga. Two methods of correction were possible: firstly, the use of a suitable chelating agent and secondly, the use of a different type of culture tank. Both methods were used, and the work is described in the following section.

Nitrogen Fixation and Nitrate Assimilation in the Presence of a Chelating Agent

Method. The chelating agent selected was ethylene diamine tetra-acetic acid (E.D.T.A.), which has a strong affinity for molybdenum. It was used in all subsequent media for experiments described in this section. This compound is a solubilizing complex-former, which cannot be metabolized itself (Schwarzenbach and Ackerman, 1948; Hutner et al., 1950). It has the disadvantage that it is not specific for molybdenum, so that it was necessary to compensate for the decreased available concentrations of other elements. Some difficulty was found in working out a satisfactorily balanced medium in which, in the presence of a sufficiently high concentration of E.D.T.A. to remove the molybdenum impurity, *Anabaena* would grow healthily.

The medium finally used had the following composition:

	Per litre of medium.
E.D.T.A.	0·05 g.
KH_2PO_4	0·2 g.
$MgSO_4\ 7H_2O$	0·2 g.
$CaCl_2$	0·1 g.
Fe (as $FeCl_3$)	10·0 mg.
Mn (as $MnSO_4$)	3·0 mg.
B (as H_3BO_3)	4·0 mg.
Cu (as $CuSO_4$)	1·0 mg.
Zn (as $ZnSO_4$)	5·0 mg.
Mo (as MoO_3)	10·0 mg.
When used:	
KNO_3	2·0 g.
NH_4Cl	1·0 g.

Although *Anabaena* grew healthily in this medium the yields were never as high as in the usual medium so that it seems probable that the optimum balance of salts was not obtained.

Using the new medium, the previous experiments were repeated. Both elemental nitrogen and potassium nitrate were used as the nitrogen source. The results of these experiments are summarized in Fig. 1. Although

maximal growth was not attained, these results clearly show that, in the presence of the chelator, little growth occurs in cultures deprived of molybdenum. The slightly convex form of the curve has not been satisfactorily explained. It remains possible that other ions might not have been present at their optimal concentrations and were retarding growth.

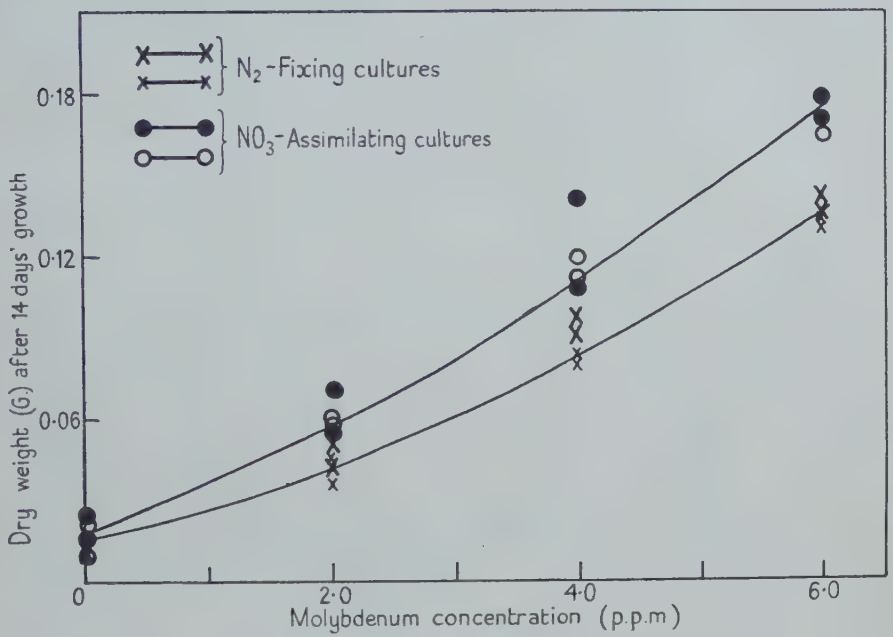

Fig. 1. Effect of molybdenum concentration on the growth of nitrogen-fixing and nitrate assimilating cultures of *A. cylindrica* in the presence of E.D.T.A.

The Effect of Molybdenum on Nitrogen Fixation and Nitrate Assimilation in 'Shake Cultures' in the Presence of E.D.T.A.

Apparatus and method

The new culture apparatus was so designed that the need for rubber tubing and cotton-wool was eliminated. It consisted of a circular perspex base, 35·5 cm. in diameter, upon which twenty 100-ml. conical flasks, each containing 30 ml. of medium, stood in two concentric circles. Each flask was covered with an inverted, 10-ml. pyrex beaker held securely in position by a thick rubber band fitted over the rim of the beaker. (As a further precaution against contamination, the insides of the beakers were coated with Dow Corning silicone fluid, DC 200, which prevented the formation of a continuous film of water from the droplets which condensed on the bases of the beakers.) The flasks were clamped in position by a second circular perspex plate, perforated with circular holes which fitted firmly over the inverted beakers, so that the plate rested upon the rubber bands covering their rims. The lower

perspex plate was immersed in water in a tank of dimensions 57 cm. × 43 cm. × 28 cm., at a depth at which the flasks were one-third submerged, and the plates were pivoted centrally so that they might rotate in a reciprocating motion through an arc of 10°, at a frequency of eighty times a minute, to facilitate the aeration of the cultures. Illumination was obtained from four submerged, 15-Watt, daylight fluorescent tubes, and the temperature of the water, which was heated by a 60-Watt soldering iron element, was thermostatically controlled at 22–23° C.

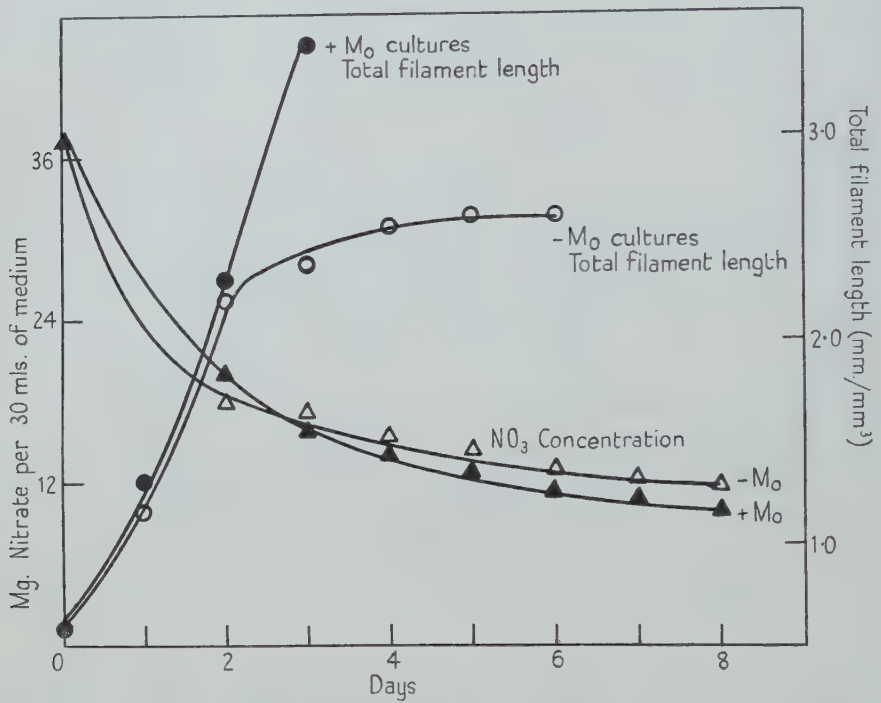

Fig. 2. The rate of growth and of nitrate absorption of nitrate assimilating cultures of *A. cylindrica* in molybdenum-deficient or normal media containing 0·05 g. per litre of E.D.T.A. Each point is the mean of triplicate determinations on duplicate samples.

This apparatus had the further advantage that it could be stopped with ease, and sample flasks removed for analysis during the exponential phase of growth. Because of the clumping of the filaments it was necessary to use the entire contents of a flask for each sampling; the clumps were broken down to free filaments by shaking the suspension with glass beads, and reliably equal quantities could then be withdrawn for replicate determinations.

In this series of experiments ten of the flasks had no molybdenum and ten of them had a molybdenum concentration of 10 p.p.m. The nitrogen source in the first experiment was potassium nitrate (2 g./litre). Daily measurements were made of total filament length and of nitrate concentration in the medium (Fig. 2). Nitrate was estimated by the Conway method (Conway, 1947). It

TABLE II

Dry Weight and Total Nitrogen of Cultures grown in the Absence of Combined Nitrogen

These results are the averages of those obtained in two separate experiments, in each of which every sample was duplicated. Each flask was inoculated (on day '0') with 1·5 mg. D.Wt. of alga, from a culture which had been transferred only once through a molybdenum-free medium.

	No molybdenum			10 p.p.m. molybdenum		
Time (days)	D.Wt. (mg. per flask)	T.N.	% T.N./D.Wt.	D.Wt. (mg. per flask)	T.N.	% T.N./D.Wt.
0	1·5	—	—	1·5	—	—
4	7·3	0·196	2·68	8·8	0·362	4·10
5	9·2	0·252	2·74	12·0	0·496	4·14
6	10·3	0·315	3·06	15·3	0·675	4·42
7	10·9	0·330	3·27	18·9	0·890	4·70

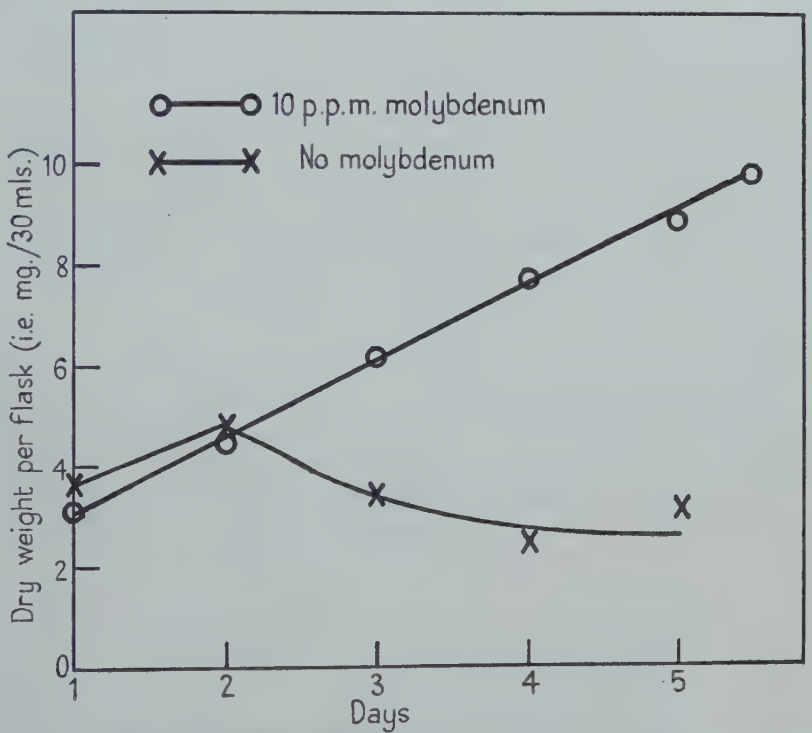

FIG. 3. The growth of nitrogen-fixing cultures of *A. cylindrica* in molybdenum-deficient and normal media containing 0·05 g. per litre of E.D.T.A. Each point is the mean of the dry weights of duplicate samples.

was reduced with Devarda's alloy and a mixture of three parts of 12 per cent. NaOH to one part of a saturated K_2CO_3 solution, and the ammonia which was formed was absorbed by N/20 sulphuric acid. The ammonia was then

Nesslerized and estimated colorimetrically by means of a Unicam Spectrophotometer. Readings were taken at 420 mμ. The ammonia which was originally present in the samples was determined separately with a saturated potassium carbonate solution, and subtracted from the 'nitrate+ammonia' results to give the actual concentration of nitrate.

In similar experiments on nitrogen fixation, growth was measured in terms of the dry weight of the cells (Table II and Fig. 3). To estimate the total nitrogen of these cells they were digested as before (p. 300), and the ammonia in the digest was determined by the Conway method, as described above.

Discussion of Results

The results are presented in Table II and in Figs. 2 and 3. It is apparent from them that the rate of nitrate uptake is unaffected by molybdenum concentration. This is in agreement with the results of similar studies by Hewitt and Jones (1947) on nitrate assimilation by molybdenum-deficient cauliflowers, and by Sakamura and Maeda (1950) with a nitrogen-fixing yeast. The block to nitrate assimilation which occurs in the absence of molybdenum cannot, therefore, be directly connected with nitrate uptake.

There is no adaptation to the deficiency of molybdenum by these cultures; the absence of deliberately added molybdenum results in the death of the alga. The initial exponential rise shown in Fig. 2 is almost certainly due to the activity of the small quantity of molybdenum contained in the inoculum, which had previously been grown in a molybdenum-supplemented medium. The later experiments, of which Fig. 3 shows a representative example, in which the inoculum had been subcultured at least three times in molybdenum-free media, show little initial increase in growth rate.

When growth is measured in terms of dry weight, the increase in dry weight is found to be significantly greater in normal than in deficient cultures and to be markedly affected by the degree of molybdenum-deficiency of the inoculum. For example, when the inoculum was derived from a first molybdenum-deficient subculture (Table II), the dry weight of cultures deprived of molybdenum had increased, 7 days after inoculation, by 627 per cent. of the original weight, whereas the dry weight of molybdenum-supplemented cultures had increased by 1,161 per cent. during this period. When the inoculum had been taken from a third molybdenum-deficient subculture (Fig. 3), the dry weight of cells grown in the absence of molybdenum had increased by only 57 per cent. in 5 days, whilst the dry weight of molybdenum-supplemented cells had increased by 336 per cent. The small amount of growth in the absence of added molybdenum leaves it possible that some fixation can occur without the element. These cultures show the usual molybdenum-deficiency symptoms of short, thin filaments with a characteristic orange-yellow tinge which would result from a decrease of phycocyanin. The occurrence of such a decrease was reported by Fogg in 1952.

A comparison of the total nitrogen:dry weight ratios for the two groups,

presented in Table II, shows that this ratio is 43·5 per cent. higher for normal than for deficient cultures, the values being 4·7 and 3·3, respectively, at the end of the experimental period, i.e. after 7 days' growth. In both cases the ratio increases during the first week of growth, but the increase is greater in the normal cultures. It is obvious from these results that the death of the alga in the absence of molybdenum is associated with a low nitrogen balance. At this stage, however, it is impossible to ascertain that this is the immediate lethal factor. It remains possible that an intermediate nitrogen compound, the conversion of which is blocked by the lack of molybdenum, accumulates in the organism to toxic concentrations.

Summary

1. Molybdenum is essential for the healthy growth of *A. cylindrica* during nitrogen fixation and nitrate assimilation, under the experimental conditions used.

2. Within the range of molybdenum concentration that it has been possible to achieve by the methods described above, molybdenum has no effect on the amount of growth when ammonium chloride is the nitrogen source.

3. Maximal growth on nitrate occurs at molybdenum levels of 0·075–0·100 p.p.m., approximately one-third of that required for maximal nitrogen fixation.

4. Insufficient molybdenum results in a low nitrogen balance in the cells.

5. Molybdenum is not required for nitrate uptake.

Acknowledgements

I am much indebted to Professor W. H. Pearsall for his encouragement, and to Dr. G. E. Fogg for stimulating and helpful advice and criticism during the carrying out of the work and in the preparation of the manuscript.

My thanks are due to the Department of Scientific and Industrial Research for their financial aid throughout the time of these investigations.

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The Effect of Molybdenum upon the Nitrogen Metabolism of *Anabaena cylindrica*

II. A More Detailed Study of the Action of Molybdenum in Nitrate Assimilation

BY

M. WOLFE

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With seventeen Figures in the Text

Abstract

Molybdenum-deficiency in *Anabaena cylindrica* results in a decrease of all the organic nitrogen fractions determined analytically with the exception of amide which occurs at approximately the same concentration irrespective of the molybdenum status. Molybdenum-rich, nitrogen-starved cells assimilate nitrate to protein rapidly in the dark; similarly treated molybdenum-deficient cells rapidly reduce nitrate to ammonia and amide, but the further synthesis to peptides and proteins proceeds very slowly. The rate of endogenous respiration is higher in deficient than in normal cells, but the rate of glycolysis is lower. No period of adaptation is required, after the addition of molybdenum to deficient cells, before normal nitrogen metabolism commences. Glucose, acetate, fumarate, succinate, and citrate are respired by normal and deficient cells in the dark. Acetate has no effect on nitrate reduction, glucose stimulates the reduction for a short period, while fumarate (and probably succinate) and, somewhat less efficiently, citrate, stimulate reduction in both normal and deficient cells and enable the deficient cells to assimilate nitrate completely to protein. It would seem that, in the absence of molybdenum, there is an insufficient supply of hydrogen and/or energy donors to permit complete nitrate assimilation.

Introduction

PREVIOUS experiments (Wolfe, 1954) have shown that molybdenum is not involved in the absorption of the nitrate ion; its possible action in a subsequent oxidation/reduction reaction remains to be investigated. Assuming this to be its function, the addition of potassium nitrate might be expected to have a different effect on the characteristics of respiration of normal and molybdenum-deficient cultures. It was therefore decided that the respiration of such cultures, in the presence and absence of potassium nitrate, would be measured by means of the standard Warburg micro-respiration manometric technique (Dixon, 1943).

Preliminary Experiments

Since no work of this nature had been done before with *Anabaena*, it was necessary to carry out preliminary experiments to see if the method was

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suitable for the alga. The organism possesses certain disadvantages in that it clumps readily and has comparatively slow growth and respiration rates. However, it was found that if the cultures were first concentrated by centrifuging at 386 g. for 5 minutes, resuspended to give a suspension containing 8–15 mg. dry weight of alga per ml., and then thoroughly shaken with glass beads to break down the clumps to free filaments, suitable material, which gave steady readings of a measurable rate of oxygen uptake, was obtained.

The experiments were carried out in a darkened tank to eliminate the occurrence of photosynthesis. The volume of carbon dioxide evolved was determined by the 'direct method' (Umbreit et al., 1949) and corrections were applied for the effect of the pH of the suspension upon the solubility of the gas (Umbreit et al., 1949).

Using a molybdenum-rich culture previously grown in a nitrate medium, the rate of oxygen uptake was measured through a pH range of pH 5·0–7·4 and through a range of phosphate buffer concentrations from M/30 to M/10. Older cultures were found to be more sensitive to the amount of phosphate used, and to the pH, than were cultures in the exponential phase of growth. For example, the respiration rate was reduced from the maximum rate of 1·95 μl. per hour per mg. dry weight, obtained at pH 7·0 with M/30 buffer, by 35 per cent. with M/15 buffer, at pH 6·8, in old cultures, compared with a reduction of 18 per cent. in young cultures. The optimal buffer concentration was found to be M/30, and the optimal pH 7·0–7·4. The Q_{O_2} of cells under these optimal conditions varied from 1·8 to 2·1 μl. per mg. dry weight of cells per hour.

The alga was found to be able to assimilate nitrate slowly for short periods (up to 24 hours—absorption continued beyond this period, but the accumulated nitrate then remained unchanged within the cells) in the dark. If the cells were nitrogen-starved overnight before being transferred to the dark, and potassium nitrate then added, the rate of assimilation was much more rapid for the first 3–5 hours; this was followed by a period of slower absorption of the nitrate and its accumulation within the cells. Prolonged dark treatment gave variable results as carbohydrate-starvation effects interfered with the normal nitrogen assimilation pattern.

Methods

Except for the initial experiments described above, and for the experiment described on p. 311, all the Warburg experiments which will be described were carried out at pH 6·0, to obviate the inaccuracies of high carbon dioxide retention by the buffer. The cultures were grown in Roux-type culture vessels in the standard potassium nitrate medium (see p. 299) with the addition or omission of molybdenum as the only variable. The light intensity was approximately 150 foot-candles in the plane of the surface of the medium, and the temperature was between 20° to 25° C. The cultures were harvested after 14–18 days' growth and the cells were centrifuged down

and washed three times, with nitrogen-free medium. The alga was then resuspended in nitrogen-free medium, at pH 7·4, and kept for 17–18 hours in an atmosphere of 78 per cent. H_2, 18·4 per cent. O_2, 2 per cent. CO_2, and 1·6 per cent. N_2 in an illuminated tank (200 foot-candles) thermostatically controlled at 25° C. In this way, the carbohydrate level was maintained whilst the nitrogen level fell sufficiently for there to be a rapid uptake of nitrate or ammonia when either of these substances was added to the medium. The cells were then centrifuged and washed again with nitrogen-free medium, transferred to Warburg flasks, and, except where otherwise stated, gassed with an 80 per cent. H_2, 20 per cent. O_2 gas mixture. The flasks were placed in a darkened Warburg tank, thermostatically controlled at 25° C., for the experiment itself.

The replacement of nitrogen by hydrogen in the Warburg flasks and in the culture bottles in which the alga was nitrogen-starved overnight is necessary to prevent the fixation of nitrogen by molybdenum-rich cultures. Hydrogen has been shown to be a specific inhibitor of nitrogen fixation in *Trifolium pratense* inoculated with *Rhizobium trifolii* (Wilson, 1940), *Azotobacter* (Wyss and Wilson, 1941), and *Nostoc muscorum* (Burris and Wilson, 1946), and, in the concentrations used, could be relied upon to inhibit the fixation of the small amounts of nitrogen present in the carbon dioxide supply.

The absence of a hydrogenase system

Hydrogen, though usually metabolically inert, is not always so, and it was necessary before proceeding further to check the possibility of the presence of a hydrogenase system in *Anabaena*.

A suspension of molybdenum-rich, nitrogen-starved cells was pipetted into Warburg flasks, one group of which were gassed with an argon:oxygen mixture, 80 per cent. A, 20 per cent. O_2, and the second group of which were gassed with an 80 per cent. H_2, 20 per cent. O_2 mixture. The endogenous rates of respiration were measured as well as the rates following the addition of a solution of potassium nitrate to give a concentration of 10 μ moles of nitrate per ml.

The results are given in Fig. 1. The rates of respiration are seen to be substantially the same in the two gas mixtures, both before and after the addition of nitrate. In both cases nitrate effected an increase in CO_2 output and a decrease in O_2 uptake, so that the respiratory quotient rose from 0·9 to 1·7. The R.Q. of control suspensions to which an equivalent volume of nitrogen-free medium was added in place of the nitrate solution remained unchanged.

From these results it can be concluded that *Anabaena cylindrica* has no hydrogenase system, or, more correctly, if such a system is present it does not operate under the experimental conditions used. The increase in the respiratory quotient of nitrogen-starved cells upon the addition of nitrate is in agreement with similar results reported by Warburg and Negelein (1920) for nitrogen-starved cells of *Chlorella vulgaris*. With *Anabaena*, however, this

change is not due solely to an increase in CO_2 output. There appears to be some competition between O_2 uptake and nitrate reduction—a finding which is more marked in later experiments in which the increase in R.Q. is due mainly, if not entirely, to the decrease in the volume of oxygen absorbed. It is considered that this might be due to the conservation of carbohydrate which is rapidly depleted during nitrate assimilation and is not replenished under these experimental conditions.

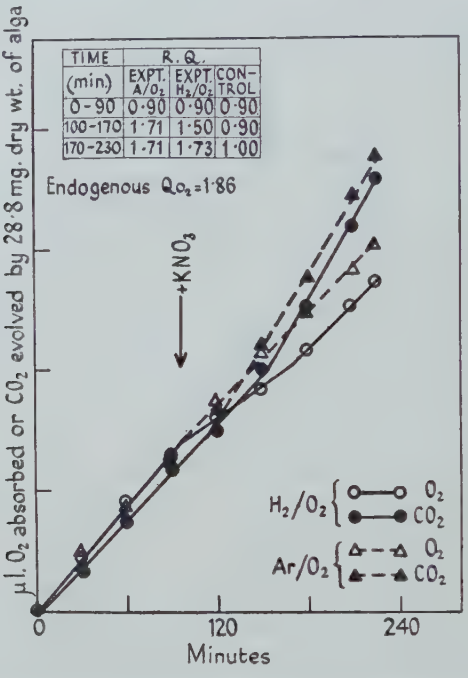


FIG. 1. The effect of the addition of 10μ moles per ml. of potassium nitrate on the respiration of normal, nitrogen-starved suspensions of *A. cylindrica* in an atmosphere of 80% H_2, 20% O_2 or 80% A, 20% O_2. Each point is the mean of duplicate determinations; readings were taken every 15 minutes, but alternate readings have been omitted here for clarity.

Effects on Nitrogen and Carbohydrate Fractions

A suspension of nitrogen-starved cells of molybdenum-rich *Anabaena*, containing 10·2 mg. dry weight per ml. was prepared in the usual way (p. 310); 2·2 ml. of the suspension and 0·5 ml. of phosphate buffer were then pipetted into each Warburg flask and gassed with 80 per cent. H_2, 20 per cent. O_2. The remainder of the suspension was divided equally into two and placed, with an equivalent volume of buffer, in conical flasks which were shaken in the same Warburg tank. Throughout the experiment 10-ml. samples were periodically withdrawn from these flasks for subsequent analysis of nitrogenous and carbohydrate fractions, so that changes in these fractions could be compared with simultaneous records of the respiration rate. After a period

of endogenous respiration (120 minutes) an equivalent amount of a nitrate solution, pH 6·0, was added simultaneously to the experimental Warburg flasks and to one of the conical flasks. The concentration of nitrate in each case was 10μ moles per ml. of suspension. At the same time an equal volume of nitrogen-free medium, pH 6·0, was added to the control Warburg flasks and to the control batch of cells in the second conical flask.

The samples taken from the conical flasks were immediately centrifuged for 5 minutes at 386 g. and the separated medium and cells stored under a thin layer of toluene in a refrigerator at 2° C. They were separately analysed later.

This experiment was repeated with cells which had been subcultured three times in a molybdenum-free, nitrate medium, prepared by the methods described in paper I.

Analytical methods. The cells were extracted with dilute trichloracetic acid and the individual fractions, with the exception of α-amino-nitrogen, estimated by the methods detailed in a paper by Syrett in 1953. The carbohydrate fractions were extracted in 70 per cent. alcohol and estimated by the modified Hagedorn–Jensen method, again as detailed by Syrett (1953).

α-Amino-nitrogen was estimated colorimetrically with a ninhydrin reagent by the method described by Moore and Stein (1948). The density of the colour was measured with a Unicam Spectrophotometer at a wavelength of 570 mμ.

Nitrate was estimated by the Conway–Nessler method previously described (see paper I).

Nitrite was estimated by the Griess–Ilosvay method, as modified by Rider and Mellon (1946).

Oxime was estimated by the modified Blom reaction, as described by Lemoigne et al. (1936).

Each determination was made in duplicate or triplicate, and, where results differed by more than about 80 μg. of nitrogen per g. dry weight of alga, the determinations were repeated.

Results

Manometric measurements (Figs. 2 and 3)

The first point of interest is the difference in the endogenous rates of respiration. The Q_{O_2} for molybdenum-deficient cells was 2·38 μl. O_2 absorbed per hour per mg. dry weight of cells, which is markedly greater than the corresponding value of 0·78 μl. for normal cells. The initial respiratory quotients, on the other hand, were similar, being between 0·9 and 1·0. Following the addition of nitrate to the suspensions, the volume of oxygen absorbed by the normal cells decreased and the respiratory quotient rose to 2·12. It remained at this level for 120 minutes, then decreased to 1·1 again. The analyses of the carbohydrate and nitrogenous fractions in the cells show that the fall in R.Q. corresponds with the point at which the polysaccharide

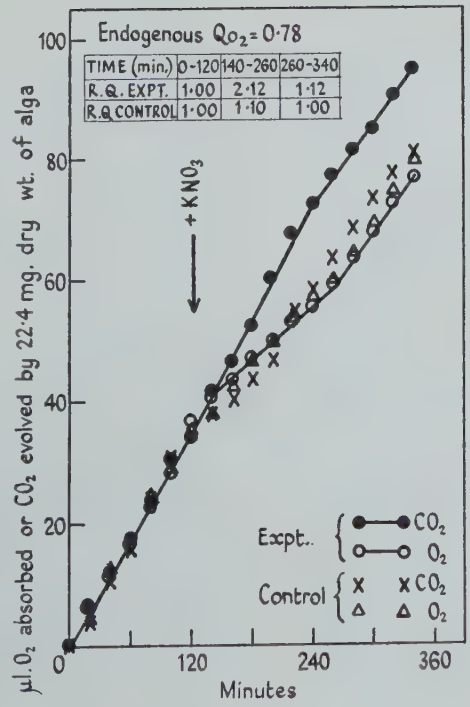


FIG. 2

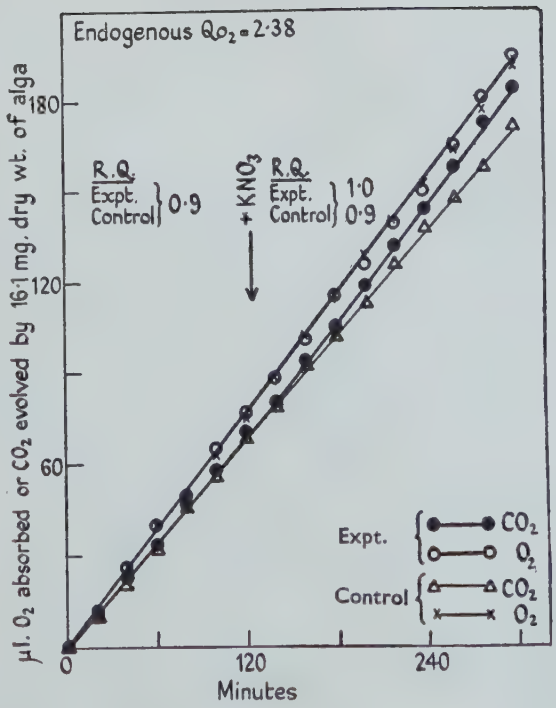
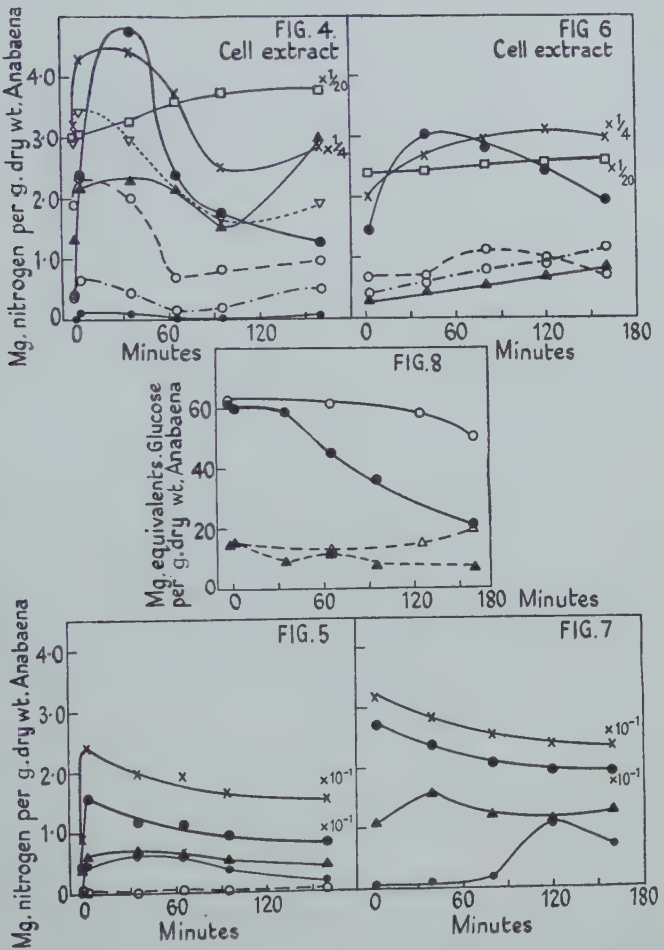


FIG. 3

FIGS. 2 and 3. The effect of the addition of 10 μ moles of potassium nitrate per ml. of suspension on the respiration of normal (i.e. molybdenum-rich), nitrogen-starved suspensions (Fig. 2.), and of molybdenum-deficient, nitrogen-starved suspensions (Fig. 3). Each point is the mean of duplicate determinations.

reserves have been reduced to a very low level and ammonia-, amide-, and α-amino-nitrogen is beginning to accumulate in the soluble nitrogen fractions.



FIGS. 4–8. The effect of the addition of potassium nitrate on the carbohydrate fractions in the cells (Fig. 8) and on the nitrogen fractions in the cells (Fig. 4) and in the medium (Fig. 5) of a normal, nitrogen-starved suspension, and on the nitrogen fractions in the cells (Fig. 6) and in the medium (Fig. 7) of a molybdenum-deficient, nitrogen-starved suspension. The initial nitrate concentration was 10 μ moles per ml.

Key: □———□ Total insoluble nitrogen. ×———× Total soluble nitrogen. ▽ ····· ▽ Combined α-amino-nitrogen. ○— —○ Free α-amino-nitrogen. ○— · —○ Amide-nitrogen. ▲———▲ Ammonia-nitrogen. ●———● Nitrate-nitrogen. • ———• Nitrite-nitrogen. ●———● Polysaccharide, expt. cells. ○———○ Polysaccharide, control cells. ▲— —▲ Disaccharide, expt. cells. Δ— —Δ Disaccharide, control cells.

With the molybdenum-deficient cells, the addition of nitrate increased the respiratory quotient only slightly—from 0·9 to 1·0.

Analytical results

A comparison of Figs. 4 and 5 with Figs. 6 and 7 brings out the following points:

1. The total nitrogen content (i.e. the sum of total soluble and total insoluble nitrogen) of molybdenum-deficient cells is lower than that of the normal cells, the values being 5·9 per cent. of the dry weight and 7·4 per cent. of the dry weight, respectively. The concentrations of all the individual nitrogen fractions which were determined are also, with the exception of amide, lower in deficient cells.

2. In both cases nitrate is rapidly absorbed and accumulates in the cells. The level then falls as the ion is metabolized.

3. In normal cells the concentration of ammonia increases rapidly at first, then decreases to its original level. The more gradual accumulation which follows is thought to be due to an insufficient supply of carbon compounds to allow complete synthesis to protein to continue.

In deficient cells the ammonia steadily accumulates.

4. Little or no nitrite appears in the cell extracts of either of these suspensions. It does, however, appear in the medium, increasing and decreasing as the nitrate is rapidly reduced.

5. No free or combined oxime was detected in the cells or in the medium of either suspension.

6. It is in the concentrations of the free α-amino-acids, soluble peptides, and amides that the differences in the two suspensions are most marked.

In normal cells there is a rapid increase of amide and of free and soluble combined α-amino-acids during the first 5 minutes after the addition of nitrate. The subsequent decrease of these fractions is associated with the increase in the insoluble nitrogen of the cells.

In molybdenum-deficient cells, on the other hand, the somewhat more gradual increase of the α-amino, amide, and soluble peptide fractions is not followed by a decrease: they remain, for the most part, accumulated in the soluble nitrogen fraction of the cells, and there is only a slow and comparatively slight increase in the insoluble nitrogen component. The increase in soluble nitrogen is predominantly in the amide fraction, which increases until all of the free α-amino-acids and, presumably, some of the combined α-amino-acids also have been converted into amides.

7. From Fig. 8 it can be seen that the assimilation of nitrate in normal cells in the dark is associated with a rapid depletion of polysaccharides. In under 2 hours over 65 per cent. of the total polysaccharide has been used for respiration and nitrate assimilation, in comparison with the 13·8 per cent. decrease in the control cells to which no nitrate had been supplied.

No analyses were made, in this experiment, of the carbohydrate fractions in the molybdenum-deficient cells, but figures for comparison can be seen in Fig. 17. Here, during the first 120 minutes of the experiment, the fall in the polysaccharide content of cells supplied with molybdenum is of the order

of 50 per cent., whereas in cells not supplied with molybdenum the fall is only 21 per cent. Deficiency of molybdenum has therefore reduced glycolycis.

The Effect of Additional Carbon Sources

It has been seen that the continued assimilation of nitrate by *Anabaena* cells kept in the dark for more than 5 hours is prevented by the depletion of the carbohydrate reserves, and that molybdenum appears to influence the linking of amino-acid synthesis with carbohydrate metabolism. It was therefore decided that certain compounds, namely glucose, acetate, fumarate, succinate, and citrate, would be tested as suitable sources to facilitate the continued assimilation of nitrate beyond this period and permit further study of this problem.

Molybdenum-rich, nitrogen-starved cells were prepared as before and suspended in Warburg flasks. After a short period of respiration in buffered, nitrogen-free medium, potassium nitrate was added, to give a concentration of 10 μ moles per ml. of suspension. After a further 2 hours the carbon compounds, each buffered at pH 6·0, were added to give initial concentrations in the medium, of 4·3 μ moles per ml. of glucose and 6·7 μ moles per ml. of each acid. The acids were added as their sodium salts.

In every case there was an increase (of up to 27 per cent.) in the rate of respiration and, except for acetate, the respiratory quotients also increased, attaining values that were higher than those which would be expected for the complete respiration of glucose or the acids alone:

Carbon source.	R.Q. for complete respiration of carbon source.	Observed R.Q. in the presence of KNO_3.
Glucose . . .	1·0	1·15
Acetate . . .	1·0	0·99
Fumarate . .	1·33	1·77
Succinate . .	1·14	1·35
Citrate . . .	1·50	1·53

Although these results suggest that fumarate and succinate, and possibly glucose and citrate, are being utilized, to some extent at least, for nitrate reduction, they do not provide definite proof.

The experiment was therefore repeated with glucose, fumarate, and citrate as the carbon sources while, simultaneously, 5·0 ml. samples were withdrawn from suspensions of similarly treated cells in conical flasks for subsequent analysis of nitrate, nitrite, and ammonia content. The results are shown in Figs. 9–11.

There was again only a small increase above the value for complete glucose oxidation of the R.Q. of cells to which glucose was added; the ratio fell to 1·15 for the first 80 minutes after the addition, and to 0·8 for the subsequent 2 hours. With both fumarate and citrate, on the other hand, the respiratory quotients rose to 1·6 and 1·67, respectively. This value was maintained in

the fumarate-respiring cells, but fell, after a further 80 minutes, in the citrate-respiring cells to 1·0.

These changes are consistent with the changes in the concentrations of nitrate and ammonia in the cells. In all cases, nitrate had begun to accumulate in the cells again before the addition of the carbon source, presumably because of insufficient available carbohydrate. In all cases, also, the carbon source effected an immediate stimulation of nitrate reduction. In the 'glucose cells', however, the nitrate began to accumulate again after 2 hours, suggesting that

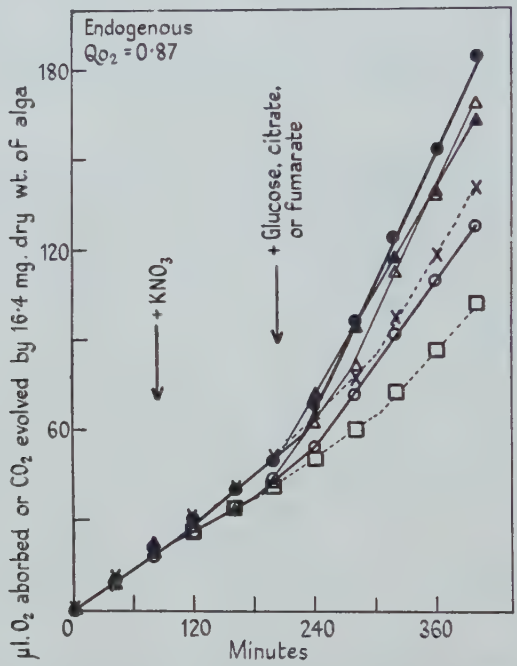
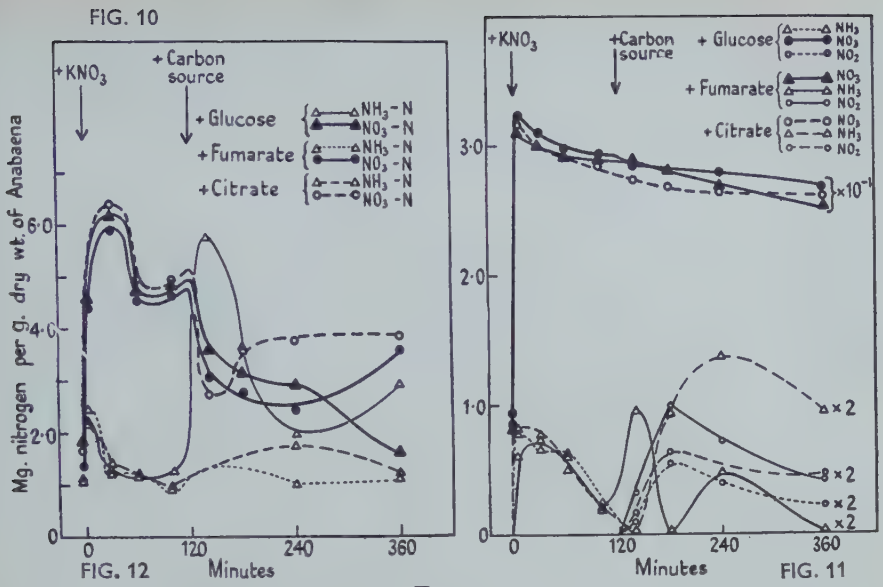
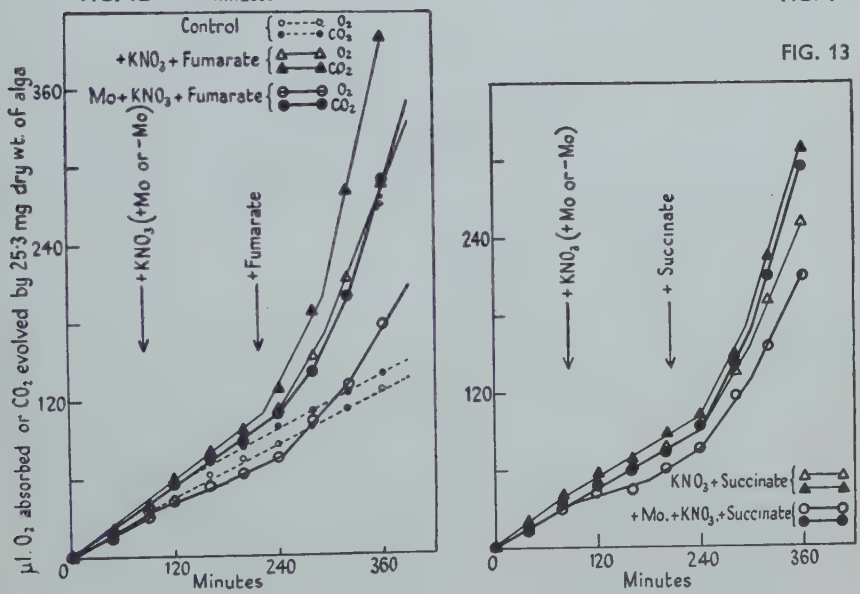


FIG. 9. The effect of the addition of 10 μ moles per ml. of potassium nitrate, followed by the addition of 1% glucose, or 6·7 μ moles per ml. of sodium fumarate or sodium citrate, on the respiration of nitrogen-starved, normal suspensions of *A. cylindrica*.

Key: +Glucose: Δ——Δ O_2; ▲——▲ CO_2. +Citrate: □----□ O_2; ×----× CO_2. +Fumarate: ○——○ O_2; ●——● CO_2.

glucose is an inadequate carbon source for nitrate assimilation. In the 'citrate cells', after an initial accumulation of nitrate, the level of the ion increased only slightly during the following 2 hours while it continued to be absorbed from the medium. It would seem, then, that citrate can be used by the cells to promote nitrate assimilation, but it is not as efficient in this respect as fumarate, which stimulated a greater absorption of nitrate from the medium and a more rapid reduction of it in the cells. The large and immediate increase in ammonia in the 'fumarate cells' is thought to be the result of the catalytic action of the acid, acting via the carboxylic acid cycle, upon the breakdown of amides.





FIGS. 10 and 11. The effect of the addition of 10 μ moles per ml. of nitrate, and of the later addition of 4·3 μ moles per ml. of glucose, or 6·7 μ moles per ml. of sodium fumarate or sodium citrate, on the nitrate, ammonia, and nitrite fractions within the cells (Fig. 10) and in the medium (Fig. 11) of a suspension of nitrogen-starved, molybdenum-rich cells.

FIGS. 12 and 13. The effect of the addition of nitrate, alone (−Mo) or with sodium molybdate (+Mo), and of the later addition of sodium fumarate (Fig. 12) or sodium succinate (Fig. 13) on the respiration of nitrogen-starved, molybdenum-deficient cells. The initial concentrations were 10 μ moles per ml. of nitrate, 6 p.p.m. of molybdenum, and 6·7 μ moles per ml. of fumarate or succinate. An equal volume of nitrogen-free medium was added each time to the control cells. Each point is the mean of duplicate determinations. Readings were taken every 20 minutes; alternate readings are omitted here for clarity.

It may be concluded from these results that fumarate and citrate can both be utilized by *Anabaena* cells to facilitate nitrate assimilation in the dark, at pH 6·0. Glucose, although it is respired by these cells, does not appear to be a suitable compound to promote continued nitrate assimilation.

The Effect of Fumaric and Succinic Acids

The aim of the first experiment in this section was twofold: firstly, to see if molybdenum functions immediately in nitrate assimilation or if an induction period is required, and, secondly, to study the effect of fumarate and succinate upon the respiration of molybdenum-deficient cells in the presence of nitrate.

A suspension of deficient cells from a third, molybdenum-free subculture, previously grown on nitrate, was nitrogen-starved overnight as usual. It was then pipetted into Warburg flasks containing nitrogen-free medium buffered at pH 6·0, and gassed with the usual 80 per cent. H_2, 20 per cent. O_2 gas mixture. After a period of endogenous respiration, potassium nitrate alone was added to the suspensions in one group of flasks, and potassium nitrate plus sodium molybdate to the second group of flasks. The initial concentrations of nitrate were 10 μ moles per ml. of suspension, and, in those flasks to which it had been added, 6 p.p.m. of molybdenum. An equal volume of nitrogen-free medium was added at the same time to the 'control flasks'. All the experimental 'carbon dioxide flasks' were duplicated. After a further 130 minutes, 20 μ moles of sodium fumarate or 20 μ moles of sodium succinate were tipped into the suspensions.

The results are given in Figs. 12 and 13.

In no case did the addition of potassium nitrate alone result in an increase in the respiratory quotient. In the presence of molybdenum, however, the respiratory quotient rose within 15 minutes to 1·7–1·8. After a further 165 minutes it fell again to 1·0–1·1, a result observed before in normal cells where nitrate assimilation has rapidly diminished the carbohydrate reserves of the cells.

The addition of fumarate again effected an increase in the R.Q. The R.Q. of cells with molybdenum increased to 1·86, and that of cells without molybdenum to only 1·32 at first, increasing to 1·46 after 2 hours (cf. the value of 1·33 for complete fumarate oxidation). Similarly with succinate, the R.Q. of cells with molybdenum increased after 30 minutes to 1·35, whilst in cells without molybdenum the R.Q. remained at 1·24; after 2 hours the respiratory quotients had increased to 1·62 and 1·40 respectively (cf. the value of 1·14 for the complete oxidation of succinate).

The increase in the respiratory quotient does not necessarily indicate the concomitant reduction of nitrate, but is in agreement with this idea. A lag period appears to be unnecessary before molybdenum can function normally.

The most interesting point from these results is the gradual increase in the respiratory quotients of the cells without molybdenum to values above those for the complete oxidation of the organic acids concerned. It seemed possible

that a process of adaptation might be taking place in which the metabolism of the organic acids was enabling the block to nitrate assimilation, resulting from the lack of molybdenum, to be by-passed.

A Further Experiment with Fumarate

To follow up this possibility, the above micro-respiration experiment was repeated with sodium fumarate as the carbon source. At the same time, suspensions of similarly treated cells were shaken in two conical flasks, in the Warburg tank, and 10-ml. samples were removed from these at intervals. The samples were centrifuged and the medium and cells were separately analysed later, as described on p. 313.

The results are given in Figs. 14–17 and they are discussed below.

1. *Manometric measurements.* As in the previous experiment, the addition of potassium nitrate resulted in an increase in the respiratory quotient only when molybdenum was simultaneously added to the cells, the values being 1·8 for cells supplied with molybdenum and 1·2 for cells without molybdenum. In both cases the addition of fumarate was followed by an increase in the rate of respiration and in the respiratory quotients, which again had values greater than that for the theoretical complete oxidation of fumarate alone.

2. *Nitrogenous fractions.* Comparing the changes in the soluble-nitrogen fractions, before the addition of fumarate, in cells to which molybdenum has been added with the nitrate with those to which nitrate has been added alone, it can be seen that molybdenum rapidly effects a difference in the metabolism of the two suspensions.

The main differences are again in the ammonia, amide, soluble-peptide, and insoluble-nitrogen fractions. In both there is a marked increase, during the first 5 minutes after the addition of nitrate, of amide nitrogen. In the cells supplied with molybdenum this is followed by the decrease of amide and an increase of both soluble, combined α-amino-nitrogen and insoluble nitrogen, whereas in cells without molybdenum the amide level remains high and there are only slight increases in peptide and insoluble nitrogen. Ammonia, which remains at approximately the same level in cells with molybdenum, increases in cells without molybdenum from 0·03 per cent. to over 1·11 per cent. of the dry weight.

The addition of fumarate results in the continued, rapid decrease of nitrate in cells without molybdenum, and in a steady increase in nitrite-, free and soluble, combined α-amino-, and insoluble-nitrogen. An hour later the amide decreases rapidly and soluble peptide and insoluble nitrogen continue to be synthesized. In cells supplied with molybdenum, nitrate assimilation is also stimulated. During the first hour and a half there is a decrease of nitrate and an increase of all the other fractions; after this, nitrate continues to be removed by further metabolism and amide, ammonia, and soluble peptide decrease whilst free α-amino-nitrogen and, more particularly, insoluble nitrogen, continue to increase.

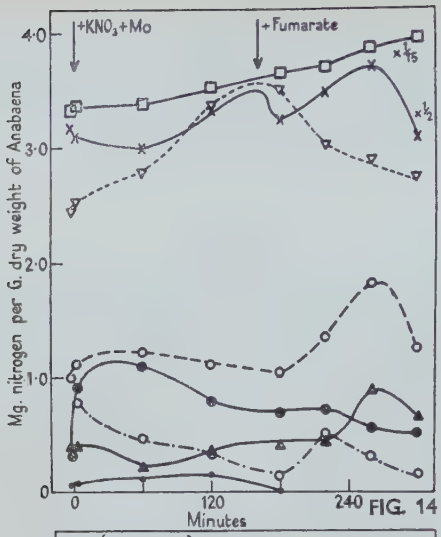


FIG. 14

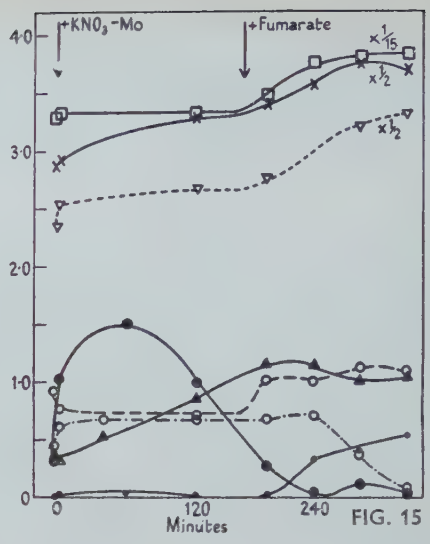


FIG. 15

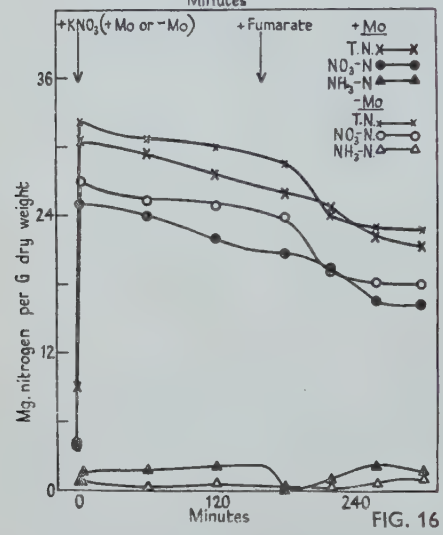


FIG. 16

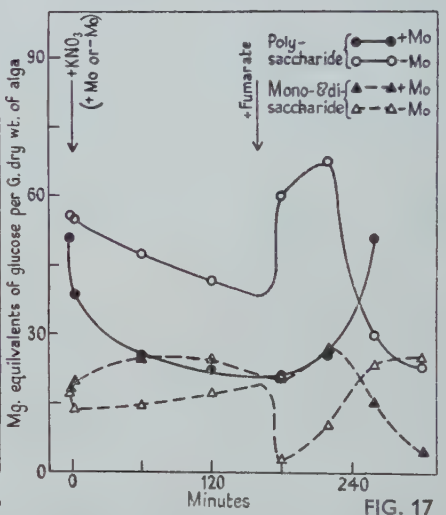


FIG. 17

FIGS. 14 and 15. The effect of the addition of nitrate alone (−Mo: Fig. 15) or with sodium molybdate (+Mo: Fig. 14), and of the later addition of sodium fumarate, on the nitrogen fractions in nitrogen-starved, molybdenum-deficient cells. The initial concentrations were 10 μ moles per ml. of nitrate, 6 p.p.m. of molybdenum, and 6·7 μ moles per ml. of fumarate.

FIG. 16. The effect of the addition of nitrate, alone (−Mo) or with sodium molybdate (+Mo), and of the later addition of sodium fumarate, on the nitrogen fractions in the media of suspensions of nitrogen-starved, molybdenum-deficient cells. The initial concentrations in the medium were: 10 μ moles per ml. of nitrate, 6 p.p.m. of molybdenum, and 6·7 μ moles of fumarate.

FIG. 17. The effect of the addition of nitrate, alone or with sodium molybdate, and of the later addition of sodium fumarate, on the carbohydrate fractions in nitrogen-starved, molybdenum-deficient cells. The initial concentrations were 10 μ moles per ml. of nitrate, 6 p.p.m. of molybdenum, and 6·7 μ moles per ml. of fumarate.

Key: □———□ Total insoluble nitrogen. ×———× Total soluble nitrogen. ▽·····▽ Combined α-amino-nitrogen. ○— —○ Free α-amino-nitrogen. ○— · —○ Amide nitrogen. ●———● Nitrate-nitrogen. ▲———▲ Ammonium nitrogen. •———• Nitrite-nitrogen.

3. *Carbohydrates.* The reduction of nitrate is accompanied, in both cells with and without molybdenum, by the catabolism of polysaccharide. The decrease is 30 per cent. greater in cells supplied with molybdenum. The addition of fumarate leads to an immediate synthesis of polysaccharide in both suspension, which is more gradual in the presence of molybdenum than in its absence, followed, an hour later, in cells without molybdenum, by a rapid decrease which coincides with the increase in insoluble nitrogen in these cells.

The changes in the nitrogen fractions and in the poly- and di-saccharides in these molybdenum-deficient cells following the addition of molybdenum and nitrate are very similar to those occurring in normal cells under the same conditions, and corroborate the idea derived from the changes in the respiratory quotients that no period of adaptation is required, after the addition of molybdenum, before normal nitrogen assimilation can commence. There is strong support, too, from the metabolic changes in cells without molybdenum after the addition of fumarate, for the suggestion that fumarate metabolism removes the barrier to rapid nitrate assimilation which otherwise exists in cells deprived of molybdenum.

General Conclusions

1. Molybdenum-deficient cells grown on nitrate characteristically have a lower nitrogen content than normal cells. This nitrogen deficiency affects all of the organic nitrogenous compounds determined analytically, with the exception of amide, which was found to occur at approximately the same concentration irrespective of the molybdenum status.

2. Nitrate is rapidly reduced to ammonia in molybdenum-deficient cells in the dark, the assimilated nitrogen accumulating mainly in the ammonia and amide fractions. The synthesis of peptides and proteins proceeds very slowly in these cells.

3. The rate of endogenous respiration is higher in molybdenum-deficient than in normal cells. The values obtained for the experiments described in this paper are:

Q_{O_2} of normal, nitrogen-starved cells: 0·83, 0·88, 0·78, 0·95.
Q_{O_2} of molybdenum-deficient, nitrogen-starved cells: 2·35, 1·48, 1·76, 2·38.

4. Fumarate, and probably succinate and citrate, stimulate the assimilation of nitrate in molybdenum-rich cells in the dark. The metabolism of these acids also enables molybdenum-deficient cells to assimilate nitrate to completion, i.e. nitrate is rapidly converted to peptides and proteins.

Discussion

These results are, in the main, consistent with those of Hewitt et al. (1949), Agarwala, Hewitt, and Jones (1950), Sakamura and Maeda (1950), Agarwala

(1951), and Agarwala and Williams (1951). Although no marked accumulation of amide is found in molybdenum-deficient cells under normal conditions (cf. Sakamura and Maeda, 1950), its concentration, proportional to the α-amino-acid content, is appreciably higher in these cells as compared with molybdenum-rich cells at a similar stage of growth. Further, when nitrate is added to deficient cells which have previously been starved of nitrogen for 18 hours, amide-nitrogen rapidly increases and the level remains high throughout the 3-hour experimental period. This is in sharp contrast with normal cells in which the newly formed amide is swiftly metabolized to peptides and proteins.

An adequate theory of the action of molybdenum must, then, account for the fact that whereas nitrate can be rapidly reduced in molybdenum-deficient cells as far as ammonia and amide while further assimilation is slow, ammonium ions supplied as chloride can be assimilated completely by these cells. It must also account for the higher rate of endogenous respiration and for the lower rate of glycolysis in molybdenum-deficient cultures. Since the addition of fumarate allows nitrate assimilation to go to completion in deficient cells, it would appear that the block to protein synthesis lies in the insufficiency of a suitable hydrogen and/or energy donor. The theoretical implications of these facts are discussed elsewhere (Fogg and Wolfe, 1954) in the light of recent work on the inhibition of phosphatases by molybdenum (for references, see Wood, 1953).

Acknowledgements

I wish to thank Dr. G. E. Fogg, under whose supervision this work was carried out, for constructive discussions and criticism of the work and of the manuscript, and Mr. P. J. Syrett and Dr. L. Fowden for helpful suggestions of analytical methods.

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The Development of the Runner-bean Leaf with Special Reference to the Relation between the Sizes of the Lamina and of the Petiolar Xylem[1]

I. The Relation between Lamina Area and Petiolar Xylem

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With four Figures in the Text

ABSTRACT

An investigation has been made of the relation between the sizes of the lamina and of the petiolar xylem of both mature and immature leaves of the runner bean (*Phaseolus multiflorus* Willd.).

The ratios xylem area/lamina area and the number of vessels/lamina area are lowest for mature leaves. Immature leaves gave higher but somewhat more variable values for these two ratios.

There is a constant growth ratio between the lamina area and the xylem area, such that k is approximately 0·61 in the allometry formula.

The significance of the results is briefly considered from the point of view that the xylem area is related to the water requirements of the leaf.

INTRODUCTION

DURING the early nineteen-hundreds much work was done on seedling anatomy from the phylogenetic point of view. Hill and de Fraine in a series of papers on seedling structure begun from this standpoint came to the conclusion that it was to physiology rather than to phylogeny that one must look for the explanation of the differences in the vascular tissue of seedlings. They observed that in general the larger seedlings had the larger amount of vascular tissue. To test this view they measured the area of the cotyledons of beech (*Fagus*) seedlings and the area cross-section of the vascular tissue in the cotyledonary stalk, and on plotting one against the other obtained a smooth curve (Hill and de Fraine, 1913). Salisbury (1913) considered the case of the green leaf. He compared the cross-sectional area of the xylem of the petiole with either the periphery (in still air) or the area (in moving air) of the lamina. Salisbury found as much as 20 per cent. divergence when making this comparison between different leaves of the same species, and as much as 3,000 per cent. when the comparison was made between leaves of different species. The idea underlying this comparison is that the size of the conducting strands is related to the size of the lamina, and thus to the rate of water loss from the leaf. His attempts to estimate the transpiration of individual leaves in order

[1] Part of a thesis approved for the degree of Ph.D. of the University of London.

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to make a direct comparison with the xylem development were unsuccessful. The ratio between the transpiration of different leaves did not remain constant when the experiment was prolonged over several days. Salisbury's conclusion, '. . . that whether we be concerned with the petiolar structure of the mature organism or with that of the cotyledons, it is to function, rather

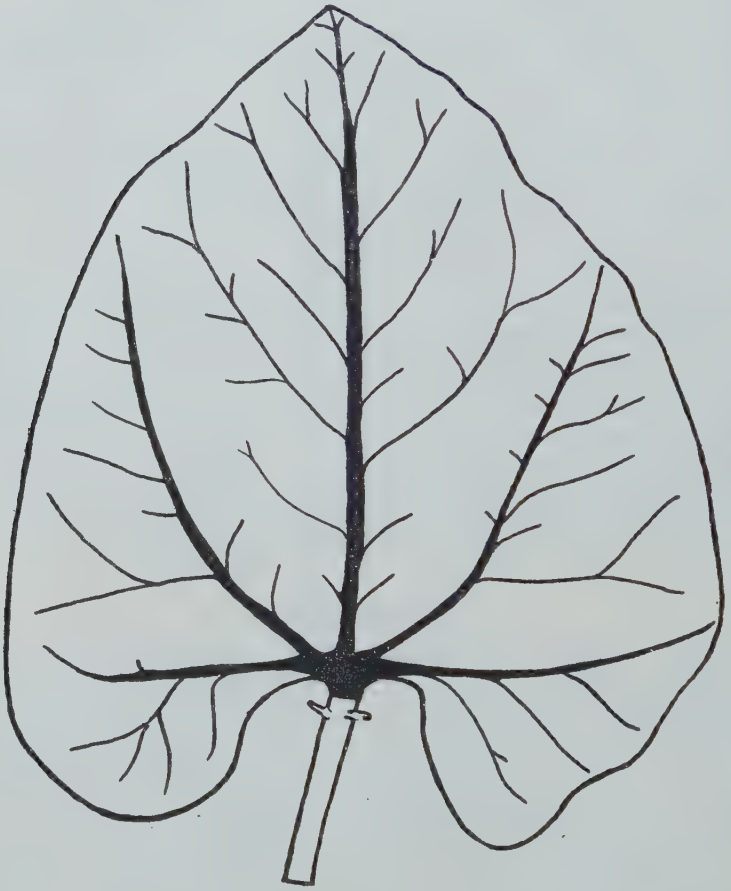

FIG. 1. A mature primary leaf of the runner bean.

than to phylogeny, that we must look for its elucidation', has been criticized by Arber (1925). She writes: 'This conclusion appears to me to be altogether too sweeping. . . . Salisbury's results do not reveal any alteration in the vascular plan of the petioles in question, or in the orientation of the bundles. The adaptational changes to which he draws attention are purely numerical and quantitative.'

The relation between petiolar xylem and lamina area is but part of the wider problem of the many correlations which exist in plants leading to the harmonious development of the various parts. A closer investigation of the rela-

tion between petiolar xylem and leaf area seemed worth while as offering a line of attack upon this wider problem.

Material

The plant chosen for this investigation was the runner bean (*Phaseolus multiflorus* Willd.). This plant offers several advantages for the present work.

1. It is easily obtainable and, most important, it is easy to grow under a wide variety of circumstances.
2. The first pair of leaves is simple and opposite: thus two leaves growing under almost identical circumstances are obtainable.
3. Secondary thickening is extremely limited in the petiole, the vascular bundles always remaining discrete.
4. The xylem is composed only of vessels and parenchyma. The parenchyma is only rarely lignified.

Mature structure of the leaf. The first pair of leaves which is already present in the embryo is simple and opposite. On either side of the stem, at right angles to and at the same level as the first pair of leaves, is inserted a pair of stipules. The lamina has stomata present on both surfaces. Water-secreting glands occur. A bundle sheath of colourless elongated cells surrounds the vascular tissue in the lamina. The petiole is grooved with a pulvinus at its upper end. Inserted just beneath the pulvinus is a pair of stipels (Fig. 1).

The Arrangement of the Vascular Tissue in the Petiole

In the embryonic petiole there are seven patches of procambial tissue arranged in the form of an arc in the body of the petiole. In addition there are two smaller patches of procambial tissue which lie one in each of the 'wings' which form the groove of the petiole. They develop to form the vascular supply to the stipels.

This arrangement of the procambial tissue is quite constant both in disposition and in number. I have not seen a single exception during the course of this work. The procambial tissue gives rise to the first formed xylem of the petiole, so that a transverse section of a young petiole shows seven vascular bundles arranged in an arc and two stipel traces (Fig. 2). There is usually a starch sheath outside each bundle. As the petiole develops there is a tendency for the xylem of each vascular bundle to open out tangentially and it may split into two masses. The phloem may or may not split. The splitting is possibly related to the necessity for maintaining an adequate presentation surface between the vessels and the surrounding parenchyma. It must not be overlooked that the xylem has to supply water to the petiole as well as to the lamina.

As development of the petiole proceeds, small secondary vascular bundles may form between the primary ones. These secondary bundles have a

similar structure to the primary ones, from which they may usually be distinguished by the absence of a starch sheath.

The vascular bundles follow a straight course through the petiole until the pulvinus is reached. Here the xylem of all the bundles becomes closely

Fig. 2

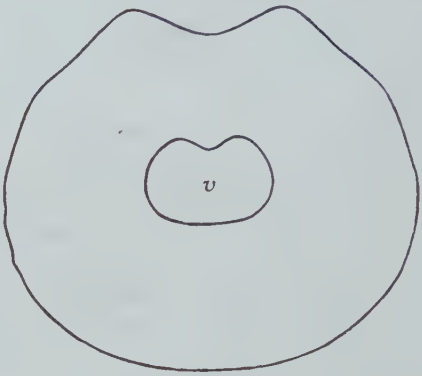

Fig. 3

Fig. 2. Diagram of a transverse section of a petiole showing the seven vascular bundles (*v*) and two stipel traces.

Fig. 3. Diagram of a transverse section of a petiole in the pulvinus region showing the aggregation of the vascular tissue (*v*).

aggregated towards the centre of the petiole (Fig. 3). Just above the pulvinus the xylem divides up to supply the lamina.

Technique and Methods

Lamina area. The laminae were cut from the petioles at the level of the stipels. The lamina was then placed upon a sheet of paper on the bench and covered with a sheet of fine ground glass, on which the outline of the lamina was traced in pencil. The outline was then traced on to paper and the area determined by means of a planimeter.

Treatment of the petiole. Each petiole was fixed for 1 hour in a mixture of three parts absolute alcohol and one part glacial acetic acid. Freehand transverse sections were cut from the upper end of the petiole at a level just basal to the stipels. The sections were stained in ammoniacal basic fuchsin, a stain which is rapid and gives good differentiation of the lignified tissue, and were mounted in Canada balsam.

Determination of the area cross-section of the xylem (*X*). The outline of the xylem vessels was traced by means of a camera lucida arranged to give a magnification of $\times 500$. The tracing was made of the inside of the wall of the vessel. The area of the xylem was then determined by means of a planimeter. The tracings of numerous vessels being joined together by single lines to give a long 'chain' so that the sum of the areas of a number of vessels was determined in one measurement. This gives added speed to a very slow and laborious process, and greater accuracy as a larger area is being measured.

Determination of vessel number (V). The number of vessels present in a petiole was found by counting from the tracings.

Determination of petiole area (P). The outline of the transverse section of the petiole was traced by means of a projection microscope arranged to give a magnification of $\times 50$. The area was then found by the use of the planimeter.

Planimetry. The planimeter was set to give areas in square centimetres. For most of this work all the planimeter measurements are the mean of 10 estimates. Two measurements were taken, the area being covered 5 times in each case. The sum of these two measurements was then divided by 10. Towards the end of the work when considerable practice had been obtained the results are the mean of three separate determinations.

Numbering of the plants. Each bean plant (B) in any series was numbered: the two leaves (P) of a plant were referred to as 1 and 2. Thus B_2P_1 refers to one leaf of the second plant, and B_2P_2 to the other leaf of the same plant.

Terms: 'Leaf' is used where the leaf as a whole is referred to. Otherwise the terms 'petiole' and 'lamina' are used in their strict sense when only part of the leaf is referred to.

Petiolar xylem area (X) is the area cross-section of the xylem tissue of the petiole.

Petiolar area (P) is the area cross-section of the petiole determined from a transverse section taken just basal to the stipels: the same transverse section from which the xylem area was found.

The Relation between Lamina Area and Petiolar Xylem

Mature leaves. The variety of bean used was Carter's 'Early Princeps'. The seed was sown in small pots (one seed to a pot) in ordinary garden soil. The pots were kept on an open balcony and were removed to the laboratory a few days before the measurements were made. At this stage the plants had formed a third leaf (the first compound leaf), and the axis was elongating rapidly. The first pair of simple leaves was, at this stage, adjudged to be mature.

The transverse sections of the petioles showed the xylem to be fully differentiated. Only in the case of one plant was an incompletely differentiated vessel seen. In many cases part at least of the protoxylem had disorganized.

The measurements made on these plants are given in Table I in the Appendix.

The ratio xylem area/lamina area (X/L), which is, in effect, the area of xylem supplying unit area of the lamina, varies from 1·9 to 4·2; the average value being 2·8. The value of this ratio for both members of any pair of leaves is generally very similar. The average difference between members of a pair is 0·24.

Similarly with the ratio number of vessels/lamina area (V/L). This varies from 2·2 to 7·5, with an average value of 4·5. The average difference in this ratio between members of a pair of leaves is 0·4.

In the light of later work, it is clear that some of the differences in the values of X/L and V/L respectively are due to developmental differences between the plants.

If the log of the lamina area is plotted against the log of the area of the petiolar xylem, the points are fitted by a straight line (Fig. 4).

That such a relationship existed between growing organs of a plant was

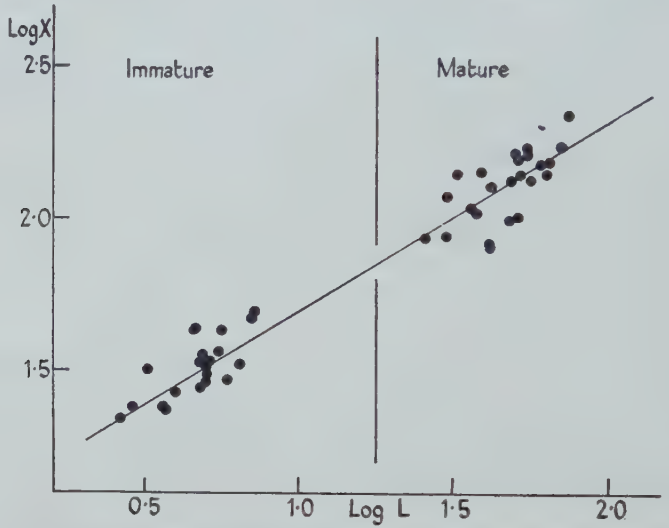

FIG. 4. Relative growth in area of xylem and lamina. Right-hand side mature leaves; left-hand side immature leaves. $k = 0{\cdot}61$.

shown by Pearsall (1927) for the stem and root weights of a number of plants, and for the lamina diameters and petiole lengths of *Tropaeolum*. Pearsall pointed out that this sort of relationship is a logical result of the fact that the growth rate of plants and of plant organs is logarithmic in character. He derived the general expression, $S = cR^k$ (the allometry formula), where c is a constant of the relative initial sizes of the organs S and R, and $k\ (= x/y)$ is the ratio of the average (relative) logarithmic growth rates, x and y, of the two organs. The value of k can be obtained either by calculation or by determining the slope of the straight line from the graph. The tangent of the angle made by the line with the x-axis gives the value of k.

Huxley (1932) developed this theme in relation to the size of an organ and the size of the body of the animal on which it is borne. k, which is the *constant differential growth ratio*, he calls the *growth coefficient of the organ*. An organ thus growing at a different rate from the body as a whole is termed *Heterogonic*. If it is growing at the same rate as the body it is termed *Isogonic*. Isogony is really a special case of heterogony.

When the figures given in Table I (Appendix) are expressed graphically (Fig. 4), there is an approximately constant differential growth ratio between the lamina area and the petiolar xylem. k is approximately 0·61 and so the growth is heterogonic.

The Relation between Petiolar Xylem and Lamina Area in Different Developmental Stages

It was clear from the observations given in the last section that other factors than lamina size affect the relationship between L and X. Therefore estimates were made of the relation between lamina area and petiolar xylem in immature (developing) leaves.

The same variety of seed was used as in the previous experiment. The plants were grown as before in pots of garden soil, but this time they were kept in the laboratory from the time of sowing, which was in February. Owing to the very low temperatures in the laboratory growth was extremely slow, and germination was rather sporadic. Examination of the transverse sections of the petioles of these immature plants showed a large number of vessel elements in the process of differentiation, while the degree to which the protoxylem had disorganized varied considerably. This rather fluid state of the vessels introduced a difficulty and a possible source of error. How could one decide when a differentiating vessel had begun to function as a pipe? And when a protoxylem element had ceased so to function? It was arbitrarily decided to consider as functional all differentiating elements which had a clearly defined lignified wall. All protoxylem elements which showed a definite lumina were considered functional.

The measurements made on these plants are given in Table II in the Appendix.

Considering the ratios X/L and V/L two points of interest emerge:

1. The wide range of values for both these ratios. X/L varies from 5·1 to 9·6 (average value 6·9); V/L varies from 10·1 to 30·3 (average value 17·6).
2. In all twenty cases these ratios are much higher than those shown in Table I (Appendix) for mature leaves, as is clear from the brief comparison below of the average values.

	Mature leaves	*Developing leaves*
X/L	2·8	6·9
V/L	4·5	17·6

Owing to the conditions under which germination and subsequent growth took place it is not unlikely that the plants were in fact of different physiological ages. These widely varying ratios may therefore represent different growth stages.

If $\log X$ is plotted against $\log L$, a similar relationship holds as for the data for the mature leaves given in Table I. The value of k is again approximately 0·61. In Fig. 4 the data for the immature leaves are shown plotted on the same graph as the data for the mature leaves. This suggests a generally similar relationship between the two sets of data.

If the varying values for the ratios X/L and V/L obtained in this experiment do in fact represent different growth stages, it would appear that these ratios are high (? maximal) at the beginning. They diminish with increasing age of

the leaf, becoming minimal at maturity. That is to say, in a mature leaf unit area of lamina is supplied by less xylem than is the case in an immature leaf.

The diminishing values of these ratios do not seem consistent with the hypothesis that xylem area is related to the water requirements of the leaf, unless it can be shown either (*a*) that the water requirements of the leaf diminish as it grows, which seems most unlikely, or (*b*) that the transporting power of the xylem is not diminishing relatively to the size of the lamina.

The elucidation of these variations of the ratios X/L and V/L, and of the points mentioned in the last paragraph, require a developmental study of the bean leaf. This will be reported in a later paper.

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Appendix

Table I

Bean plant.	Lamina area in sq. cm. *L.*	Xylem area in sq. cm. × 500 *X.*	Number of vessels *V.*	Ratio *X/L.*	Ratio *V/L.*
B_1P_1	41·47	80·82	92	1·90	2·2
B_1P_2	41·52	80·11	106	1·92	2·6
B_2P_1	36·80	109·06	178	2·96	4·6
B_2P_2	35·95	107·73	172	2·99	4·8
B_3P_1	55·21	159·16	203	2·88	3·7
B_3P_2	54·92	169·58	237	3·10	4·3
B_4P_1	41·26	125·51	209	3·04	5·0
B_4P_2	39·04	142·01	204	3·63	5·2
B_5P_1	30·17	118·59	215	3·96	7·1
B_5P_2	32·40	139·05	244	4·28	7·5
B_6P_1	30·39	88·08	152	2·89	5·0
B_6P_2	25·99	85·01	173	3·31	6·7
B_7P_1	70·66	171·39	259	2·42	3·7
B_7P_2	76·28	212·85	294	2·73	3·8
B_8P_1	49·66	160·73	228	3·23	4·6
B_8P_2	51·07	155·08	242	3·03	4·7
B_9P_1	64·07	150·60	226	2·35	3·5
B_9P_2	62·77	136·86	242	2·18	3·8
$B_{10}P_1$	52·42	137·69	206	2·60	3·9
$B_{10}P_2$	56·21	132·59	188	2·36	3·3
$B_{11}P_1$	48·12	97·10	189	2·02	3·9
$B_{11}P_2$	51·17	99·12	227	1·93	4·4
$B_{12}P_1$	48·44	132·26	225	2·73	4·6
$B_{12}P_2$	59·79	146·28	253	2·44	4·5
Average values	48·16 sq. cm.	130·7	207	2·8	4·5

TABLE II

Bean plant.	Lamina area in sq. cm. *L.*	Xylem area in sq. cm. × 500 *X.*	Number of vessels *V.*	Ratio *X/L.*	Ratio *V/L.*
B_1P_1	7·29	48·81	88	6·7	12·0
B_1P_2	5·67	42·76	77	7·5	13·5
B_2P_1	7·11	46·53	72	6·5	10·1
B_2P_2	5·16	33·82	76	6·5	14·6
B_3P_1	4·58	43·52	70	9·5	15·2
B_3P_2	3·27	31·60	100	9·6	30·3
B_4P_1	2·60	22·12	59	8·5	22·8
B_4P_2	2·86	24·04	56	8·4	19·3
B_5P_1	5·83	29·76	73	5·1	12·6
B_5P_2	6·41	33·06	105	5·2	16·4
B_6P_1	5·02	32·16	89	6·4	17·8
B_6P_2	4·00	26·82	76	6·7	19·0
B_7P_1	4·74	34·02	73	7·2	15·5
B_7P_2	5·45	36·58	117	6·7	21·3
B_8P_1	5·01	28·76	98	5·7	19·6
B_8P_2	4·85	35·57	103	7·3	21·0
B_9P_1	4·67	31·25	74	6·6	15·7
B_9P_2	4·81	27·68	68	5·7	14·2
$B_{10}P_1$	3·68	23·30	66	6·3	17·8
$B_{10}P_2$	3·60	23·72	82	6·5	22·8
Average values	4·83 sq. cm.	32·79	81	6·9	17·6

The Development of the Runner-bean Leaf with Special Reference to the Relation between the Sizes of the Lamina and of the Petiolar Xylem[1]

II. The Normal Development of the Bean Leaf

BY

D. J. B. WHITE

(*Department of Botany, University College, London*)

With seven Figures in the Text

Abstract

The development of the first pair of leaves of the Runner-bean (*Phaseolus multiflorus* Willd.) has been investigated from the unfolding of the leaves to the end of lamina expansion. The growth of the petiole is relatively fast and ceases first. Extension growth of the lamina continues longer. In the growth of the xylem, the average area of the vessels formed increases most markedly at an early developmental stage. Vessel number may continue to increase after lamina growth has ceased. Both the number of vessels and the xylem area per unit area of lamina diminish as the leaf matures.

Introduction

IN the first paper in this series an account was given of the relation between lamina area and petiolar xylem area of mature and immature leaves of *Phaseolus multiflorus* Willd. It was found that the ratios xylem area/lamina area (X/L) and number of vessels/lamina area (V/L) were highest for the immature leaves (White, 1954). The results suggested that the values of these ratios varied as the leaf increased in size and it was therefore decided to follow the changes developmentally.

There is an obvious difficulty in doing this. While it is possible to make, say, daily measurements of petiole length or lamina area of the same leaf, it is possible to make only one determination of petiolar xylem on any one leaf. The method adopted therefore was to grow a large batch of plants and to remove samples of 5 plants at intervals. Xylem area, lamina area, &c., were measured for each plant, thus giving 10 values for each feature measured for the 5 plants removed at each stage. The average value for each sample could then be found.

This involved the problem of getting a large number of seedlings all at the same stage of development. Even when this is successfully achieved minor variations may occur during subsequent development due either to intrinsic differences in the seedlings or to minor variations in the environment.

[1] Part of a thesis approved for the degree of Ph.D. of the University of London.

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Inspection of the detailed measurements given in the Appendix will show examples of plants whose dimensions suggest that they belong more properly either to the preceding set or to the following one, i.e. they are plants whose development has either lagged or shot ahead.

Material and Methods

The variety of bean used was Carter's 'Streamline' 1947 crop. About 600 seeds were roughly selected on the basis of seed size. After soaking in tap-water for 24 hours they were planted in damp sawdust on July 1. On July 17 the seedlings, which by now had well-developed root systems but with the first pair of leaves still unfolded, were transplanted into pots of soil. Three days later 200 seedlings were selected as being in the same stage of development as far as visual inspection could reveal. Five of these plants were removed and fixed ('Control Sample 0'). Fifty plants[1] untreated in any way were allowed to grow on. Five plants were removed on July 22, 24, 26, 28, 30, and August 3. The plants removed on each occasion are referred to as Control Samples, 1, 2, &c.

For each plant the following determinations were made by the methods described in an earlier paper (White, 1954):

1. Length and area cross-section of the petiole.
2. Area of the petiolar xylem.
3. The number of vessels in the transverse section of the petiole.
4. The length, breadth, and area of the lamina.

The details of the measurements made on all these plants are given in the tables in the Appendix. From these details certain conclusions may be drawn as to the normal development of the first leaf of this plant. (In the text the average (group) values have been used and the results expressed graphically.)

The Normal Development of a Bean Leaf

This involves the detailed description of the development of three features separately: (*a*) the lamina, (*b*) the petiole, and (*c*) the xylem, and a consideration of the relations between them.

1. *Lamina*

(*a*) *Growth in area.* The lamina area when plotted against time (in Fig. 1) gives a normal type of growth curve between areas of approximately 6 sq. cm. to 63 sq. cm. The lamina had reached half its final size on about the fifth day.

(*b*) *Growth in length and breadth.* The curves for growth in length and breadth of the lamina are shown in Fig. 2. The two curves take a parallel course. The ratio lamina length/lamina breadth (l/b) remains constant (about 1·2) during development. This is illustrated in Fig. 3, where lamina length has been plotted against lamina breadth. There is thus a simple and

[1] The remainder of the plants received varying treatments, the results of which will be considered in the final paper of this series.

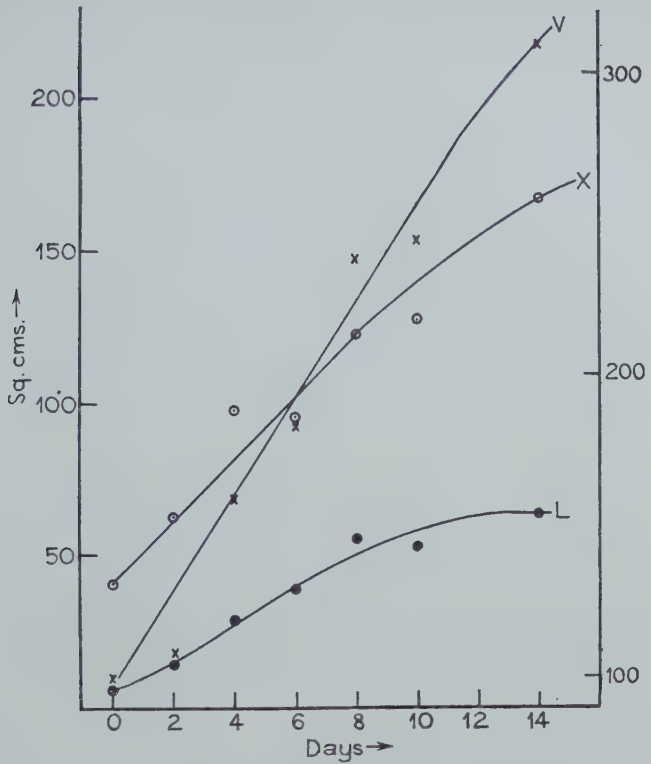

FIG. 1. Growth curves for lamina area (*L*), xylem area (*X*), and vessel number (*V*). The vertical scale for *V* is on the right.

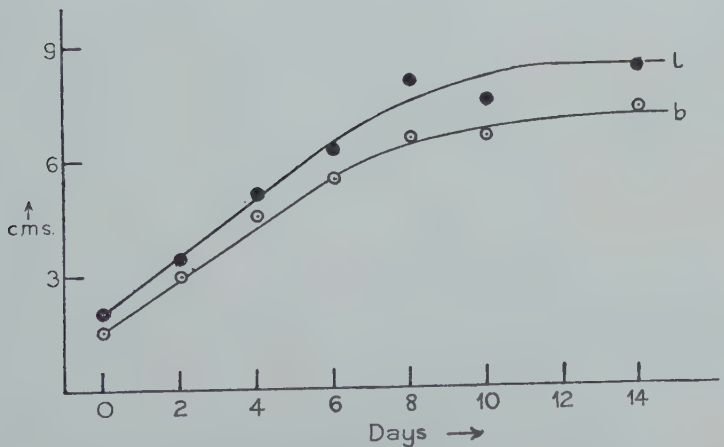

FIG. 2. Growth curves for lamina length (dots) and lamina breadth (circles).

direct relationship between these two dimensions of the lamina. No material variation in this condition has been observed in any series or as a result of any treatment.

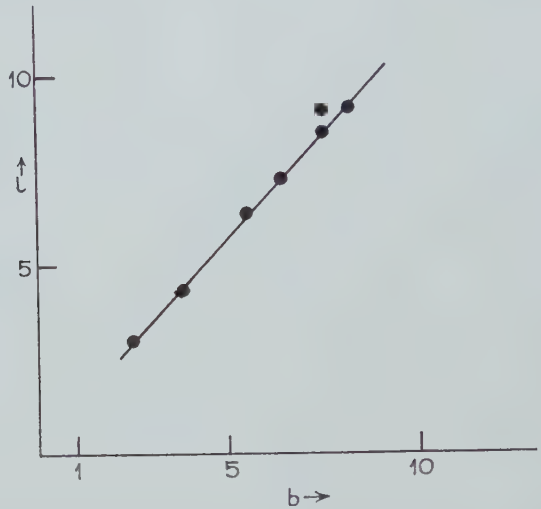

FIG. 3. Lamina length (*l*) plotted against lamina breadth (*b*).

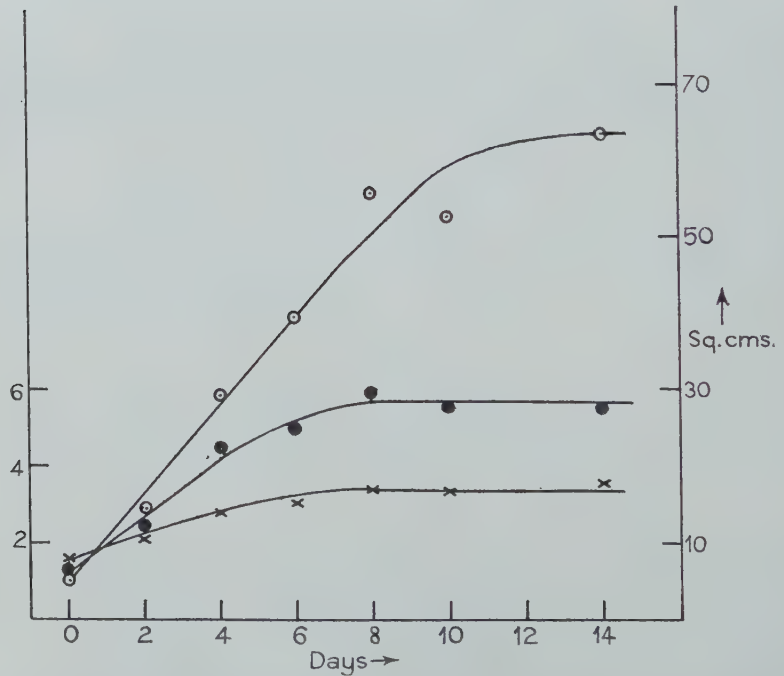

FIG. 4. Growth curves for petiole length (dots) and petiole area (crosses). Curve for lamina area (circles) shown for comparison. Vertical scale for lamina area on the right.

2. *Petiole*

(*a*) *Growth in length.* The growth curve for petiolar length is shown in Fig. 4. Rapid growth occurred during the first 6–7 days, by which time growth in length was almost complete. In Fig. 4 the growth curve for lamina area has been plotted on the same time-scale, and comparison of this curve with that for petiole length shows that the growth of the petiole in length stops some days before the growth of the lamina ceases. It can be seen from the graph that the petiole is half-grown by the second day, whereas the lamina does not reach half its final area until the fourth or fifth day.

(*b*) *Growth in cross-sectional area.* The petiole grew in cross-sectional area more slowly than it grew in length during the first 6 days (Fig. 4). The duration of growth is similar in both cases. The curves shown in Fig. 4 illustrate the comparative rapidity of the development which seems to be characteristic of the petiole.

3. *Xylem development in the petiole*

(*a*) *Cross-sectional area* (*X*). The growth curve for the area of the xylem is shown in Fig. 1. Increase in xylem area continues, at least for a time, after the lamina growth in area has ceased.

(*b*) *Vessel number* (*V*). Vessel number has been plotted against time on the same graph (Fig. 1). Rate of increase in vessel number was at first slow, but the number of vessels continues to increase even *after* lamina growth has ceased.

(*c*) *Average area of the vessels.* The present study confirms the suggestion obtained from some earlier experiments that there is a rapid increase, at an early stage, in the average area of the vessels, which afterwards remain more or less constant in size. The average areas of the vessels of the plants under consideration at the moment are shown graphically in Fig. 5. The first set of plants gave the lowest values for average vessel size ($7{\cdot}9 \times 10^{-4}$ sq. cm.). Increase in the average vessel area had taken place by the second day, and thereafter the value fluctuated about a mean of $11{\cdot}3 \times 10^{-4}$ sq. cm. These later fluctuations are not statistically significant.

As may be seen from Fig. 5, this marked increase in vessel area takes place before the lamina has reached half its final size, and almost entirely precedes the main phase of increase in vessel number (cf. Fig. 1).

4. *The relationship between xylem area and lamina area*

(*a*) *The ratio X/L.* The value of this ratio for Control Sample 0 (the youngest and most immature leaves) was 7·1. The value of the ratio fell steadily (Fig. 6) with only minor fluctuations. The minimum value was about 2·2. Thus the ratio of xylem area to lamina area decreases during development; or to put it another way, at maturity a unit of xylem serves a greater area of lamina than at any earlier stage.

(*b*) *The relative growth of the xylem area* (*X*) *and the lamina area* (*L*). Log *X* plotted against log *L* yields approximately a straight line (Fig. 7).

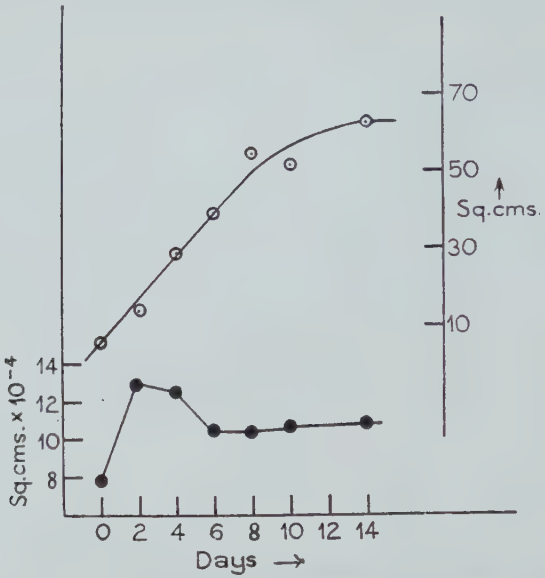

FIG. 5. Average area of the vessels plotted against time (dots). Curve for lamina area (circles) shown for comparison. Vertical scale for lamina area on the right.

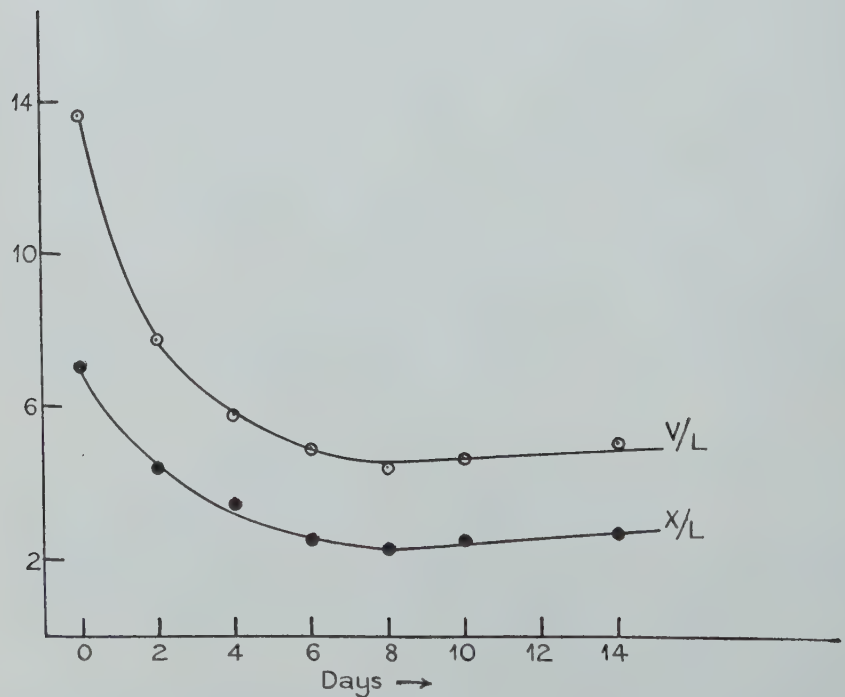

FIG. 6. The ratios V/L and X/L.

Statistically the data are adequately fitted by a straight line with k approximately 0·67 (compared to 0·61 obtained for an earlier series (White, 1954)).

This is another way of saying that the lamina grows faster than does the xylem. This logarithmic relationship can only be an approximate expression of the over-all growth relations between the areas of the xylem and the lamina. A constant differential growth ratio can only exist between the xylem area and the lamina area while both are increasing (cf. Pearsall, 1927; Huxley, 1932). There is evidence (Fig. 1) that increase in xylem area continues after lamina extension has ceased. Nevertheless the logarithmic plotting provides a useful means of illustrating the nature of the relationship between the xylem area and lamina area while *both* are still growing and for comparing the relationship under various conditions.

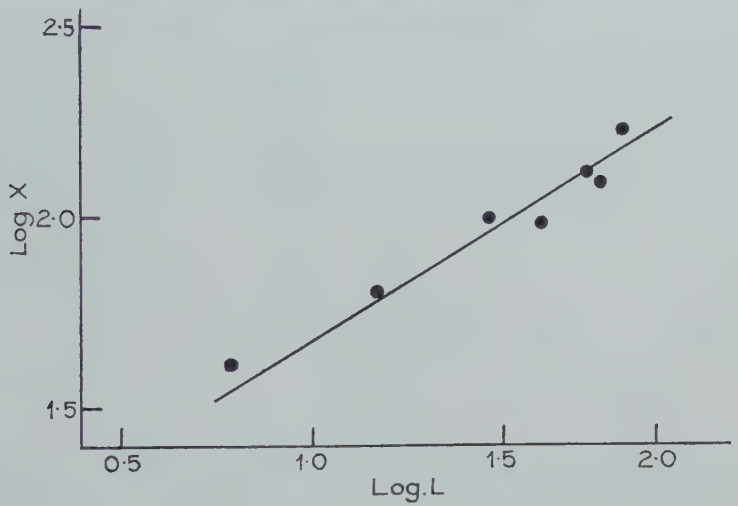

Fig. 7. Relative growth in area of xylem and lamina. $k = 0{\cdot}67$.

5. *Relationship between vessel number (V) and lamina area (L)*

The ratio V/L. In the main the ratio V/L follows a course similar to the ratio X/L. It is maximal in the least mature stages (about 13·6) and tends to become minimal at maturity (about 5·0). That is, as the leaf approaches maturity a unit of lamina is supplied by fewer vessels. There is in fact a slight rise in the value of the ratio towards the end of development since the number of vessels was still increasing when lamina extension had ceased. (Fig. 6.)

6. *Relationship between xylem development and petiole development*

The development of the petiole is rapid, being completed by about the sixth day, while the development of the xylem is continued longer. Thus, as development of the leaf takes place a greater proportion of the petiole is occupied by xylem tissue. This state of affairs is to be seen in all the plants irrespective of the treatment they received.

This work confirms the suggestion made previously (White, 1954) as to

the difficulty of regarding the development of the lamina in such leaves as dependent upon water-supply through the xylem as measured by xylem area or vessel number.

Further discussion of these results is left to the final paper in this series, when data from further work to be described can be taken into account.

Acknowledgement

I am indebted to Mr. N. W. Please of the Department of Statistics, University College, London, who examined my data statistically for me.

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APPENDIX: DATA

Table I

Sample o

Bean plant.	Lamina area in sq. cm. L.	Xylem area in sq. cm. $\times$ 500. X.	Number of vessels V.	Average area of vessels sq. cm. $\times 10^{-4}$.	Ratio X/L.	Ratio V/L.
B_1P_1	7·99	46·51	123	7·6	5·8	15·38
B_1P_2	6·69	43·51	114	7·2	6·95	17·02
B_2P_1	6·72	64·09	133	9·8	9·6	19·79
B_2P_2	5·99	52·63	88	11·9	8·99	14·69
B_3P_1	4·59	52·03	124	8·4	11·33	27·02
B_3P_2	4·57	32·41	99	6·5	7·08	21·66
B_4P_1	7·5	25·28	73	6·9	3·37	9·73
B_4P_2	7·96	30·9	83	7·4	3·38	10·42
B_5P_1	3·91	25·97	76	6·8	6·64	19·44
B_5P_2	4·19	32·61	95	6·9	7·78	22·66
Average	6·01	40·59	100	7·9	7·1	13·6

Bean plant.	Lamina		Ratio l/b.	Petiole		X as percentage of P.
	Length (l).	Breadth (b)		Length.	Area (P)	
B_1P_1	3·3	2·7	1·22	1·2	1·52	6·1
B_1P_2	2·8	2·6	1·07	1·0	1·22	7·1
B_2P_1	3·6	2·7	1·39	1·7	1·47	8·7
B_2P_2	3·2	2·6	1·26	1·5	1·48	7·1
B_3P_1	2·6	2·3	1·13	1·0	1·13	9·0
B_3P_2	2·7	2·0	1·35	1·0	1·36	4·8
B_4P_1	3·6	2·6	1·38	1·5	1·34	3·8
B_4P_2	3·4	3·0	1·14	1·5	1·47	4·2
B_5P_1	2·5	2·1	1·21	1·1	0·99	5·2
B_5P_2	2·6	2·0	1·3	1·1	1·00	6·5
Average	3·03	2·46	1·25	1·3	1·3	6·3

Table II

Sample 1

Bean plant.	Lamina area in sq. cm. L.	Xylem area in sq. cm. × 500 X.	Number of vessels V.	Average area of vessels sq. cm. × 10^{-4}.	Ratio X/L.	Ratio V/L.
B_1P_1	15·04	53·90	110	9·8	3·58	7·31
B_1P_2	13·36	47·35	101	9·4	3·55	7·57
B_2P_1	13·62	74·13	135	11·0	5·44	9·91
B_2P_2	12·68	47·31	130	7·3	3·73	10·25
B_3P_1	14·53	71·25	109	13·1	4·90	7·50
B_3P_2	16·55	77·85	90	17·3	4·70	5·44
B_4P_1	15·83	77·46	119	13·0	4·89	7·52
B_4P_2	11·23	69·18	84	16·5	6·16	7·48
B_5P_1	16·31	59·54	114	10·4	3·65	6·99
B_5P_2	15·18	49·76	89	22·5	3·28	7·38
Average	14·43	62·8	108	13·0	4·4	7·7

Bean plant.	Lamina: Length (l).	Lamina: Breadth (b).	Ratio l/b.	Petiole: Length.	Petiole: Area (P).	X as percentage of P.
B_1P_1	4·6	4·0	1·17	2·0	1·36	7·9
B_1P_2	4·4	3·9	1·13	1·9	1·3	7·3
B_2P_1	4·3	3·5	1·23	2·0	1·99	7·4
B_2P_2	4·1	3·7	1·11	1·9	1·78	5·3
B_3P_1	4·3	3·9	1·10	3·0	2·08	6·9
B_3P_2	4·9	3·7	1·32	3·2	2·42	6·4
B_4P_1	4·6	3·8	1·2	2·5	2·04	7·6
B_4P_2	3·6	3·3	1·09	2·2	2·29	6·0
B_5P_1	4·4	4·4	1·0	2·4	2·25	5·3
B_5P_2	4·0	4·3	0·93	2·7	1·98	5·0
Average	4·3	3·9	1·13	2·4	1·95	6·5

Table III

Sample 2

Bean plant.	Lamina area in sq. cm. L.	Xylem area in sq. cm. × 500 X.	Number of vessels V.	Average area of vessels sq. cm. × 10^{-4}.	Ratio X/L.	Ratio V/L.
B_1P_1	31·14	81·99	157	10·4	2·63	5·04
B_1P_2	23·31	68·63	127	10·8	2·94	5·45
B_2P_1	30·87	97·31	146	13·3	3·15	4·73
B_2P_2	29·75	120·3	144	16·7	4·04	4·84
B_3P_1	41·41	112·6	154	14·6	2·72	3·72
B_3P_2	42·95	115·8	186	12·5	2·70	4·33
B_4P_1	24·24	76·61	177	8·7	3·16	7·30
B_4P_2	21·03	70·01	137	10·2	3·33	6·51
B_5P_1	22·98	124·7	195	12·8	5·43	8·49
B_5P_2	21·59	114·4	168	13·6	5·30	7·78
Average	28·93	98·23	159	12·4	3·54	5·82

Bean plant.	Lamina: Length (l).	Lamina: Breadth (b).	Ratio l/b.	Petiole: Length.	Petiole: Area (P).	X as percentage of P.
B_1P_1	7·1	5·7	1·25	3·5	2·79	5·9
B_1P_2	5·9	4·5	1·31	3·5	2·13	6·4
B_2P_1	5·7	6·0	0·95	4·3	2·20	8·8
B_2P_2	5·7	5·9	0·97	4·5	2·38	10·1
B_3P_1	7·2	7·0	1·03	5·5	2·45	9·2
B_3P_2	7·4	6·3	1·17	5·2	2·91	8·0
B_4P_1	6·2	5·1	1·22	4·3	2·69	5·7
B_4P_2	5·7	4·8	1·19	4·0	2·52	5·6
B_5P_1	6·4	4·7	1·36	5·0	3·59	7·0
B_5P_2	5·6	5·1	1·10	5·2	3·20	7·2
Average	6·3	5·5	1·16	4·5	2·69	7·4

Table IV

Sample 3

Bean plant.	Lamina area in sq. cm. L.	Xylem area in sq. cm. × 500 X.	Number of vessels V.	Average area of vessels sq. cm. × 10^{-4}.	Ratio X/L.	Ratio V/L.
B_1P_1	42·97	105·2	154	13·7	2·45	3·58
B_1P_2	40·97	85·39	161	10·6	2·08	3·93
B_2P_1	31·30	106·7	187	11·4	3·34	5·97
B_2P_2	31·53	118·3	197	12·0	3·75	6·25
B_3P_1	47·60	114·2	186	12·3	2·40	3·91
B_3P_2	32·80	84·89	148	11·5	2·59	4·51
B_4P_1	53·17	91·53	222	8·2	1·72	4·18
B_4P_2	40·50	82·83	207	8·0	2·05	5·11
B_5P_1	30·20	82·76	179	9·2	2·74	5·93
B_5P_2	36·57	84·70	193	8·8	2·32	5·28
Average	38·77	95·59	183	10·6	2·54	4·87

Bean plant.	Lamina		Ratio l/b.	Petiole		X as percentage of P.
	Length (l).	Breadth (b).		Length.	Area (P).	
B_1P_1	7·6	7·2	1·06	5·5	2·62	8·03
B_1P_2	7·2	6·3	1·14	5·7	2·71	6·30
B_2P_1	6·5	5·8	1·12	5·1	2·53	8·44
B_2P_2	7·3	5·2	1·40	5·6	2·78	8·51
B_3P_1	7·8	7·8	1·0	4·7	2·94	7·77
B_3P_2	6·1	6·1	1·0	2·5	2·59	6·56
B_4P_1	8·8	7·0	1·26	4·1	3·41	5·37
B_4P_2	7·2	6·9	1·04	4·3	2·92	5·67
B_5P_1	6·2	6·2	1·0	4·5	3·11	5·45
B_5P_2	7·1	5·7	1·25	4·3	3·17	5·36
Average	7·18	6·42	1·13	4·9	2·88	6·75

Table V

Sample 4

Bean plant.	Lamina area in sq. cm. L.	Xylem area in sq. cm. × 500 X.	Number of vessels V.	Average area of vessels sq. cm. × 10^{-4}.	Ratio X/L.	Ratio V/L.
B_1P_1	70·57	120·5	272	8·9	1·71	3·85
B_1P_2	65·97	118·9	238	10·0	1·80	3·61
B_2P_1	73·47	181·6	332	10·9	2·47	4·52
B_2P_2	76·90	170·9	300	11·4	2·22	3·90
B_3P_1	38·57	101·1	218	9·3	2·62	5·65
B_3P_2	43·13	100·6	196	10·3	2·33	4·54
B_4P_1	49·30	97·6	191	10·2	1·98	3·87
B_4P_2	50·33	95·03	191	10·0	1·89	3·79
B_5P_1	46·70	119·1	252	9·5	2·55	5·40
B_5P_2	43·30	124·2	198	12·5	2·87	4·57
Average	55·82	123·0	238	10·3	2·24	4·37

Bean plant.	Lamina		Ratio l/b.	Petiole		X as percentage of P.
	Length (l).	Breadth (b).		Length.	Area (P).	
B_1P_1	10·7	8·6	1·24	5·5	3·23	7·46
B_1P_2	9·5	8·7	1·09	5·4	2·84	8·37
B_2P_1	10·0	8·7	1·15	6·2	3·75	9·68
B_2P_2	9·4	8·9	1·06	6·2	4·19	8·16
B_3P_1	7·6	6·8	1·12	6·3	3·23	6·26
B_3P_2	8·4	5·8	1·45	6·3	3·61	5·57
B_4P_1	8·8	7·3	1·21	6·2	3·26	5·99
B_4P_2	9·3	6·8	1·37	5·8	2·81	6·77
B_5P_1	8·2	6·9	1·19	5·2	2·96	8·05
B_5P_2	7·7	6·8	1·13	6·0	2·48	10·0
Average	8·96	7·53	1·20	5·9	3·24	7·63

Table VI

Sample 5

Bean plant.	Lamina area in sq. cm. *L.*	Xylem area in sq. cm. × 500 *X.*	Number of vessels *V.*	Average area of vessels sq. cm. × 10^{-4}.	Ratio *X*/*L.*	Ratio *V*/*L.*
B_1P_1	54·93	148·0	263	11·3	2·69	4·79
B_1P_2	59·40	159·9	312	10·3	2·69	5·25
B_2P_1	47·5	111·9	277	8·1	2·36	5·83
B_2P_2	49·37	120·8	191	12·7	2·45	3·87
B_3P_1	54·30	142·3	275	10·4	2·60	5·02
B_3P_2	61·73	138·3	233	11·9	2·24	3·78
B_4P_1	55·03	107·5	258	8·3	1·95	4·69
B_4P_2	56·03	111·3	282	7·9	1·99	5·03
B_5P_1	38·40	114·0	174	13·1	2·97	4·53
B_5P_2	48·40	124·7	175	14·3	2·58	3·62
Average	52·51	127·9	244	10·8	2·45	4·64

Bean plant.	Lamina		Ratio *l*/*b.*	Petiole		*X* as percentage of *P.*
	Length (*l*).	Breadth (*b*).		Length.	Area (*P*).	
B_1P_1	8·3	8·3	1·0	5·8	3·34	8·88
B_1P_2	8·5	7·2	1·18	5·5	3·30	9·69
B_2P_1	9·1	7·2	1·26	6·5	2·98	7·51
B_2P_2	9·1	7·1	1·28	5·2	2·71	8·92
B_3P_1	9·2	7·4	1·24	5·0	3·56	8·00
B_3P_2	7·3	9·0	0·81	5·2	3·86	7·17
B_4P_1	8·8	7·4	1·19	5·7	2·86	7·52
B_4P_2	9·1	7·4	1·23	5·4	3·42	6·51
B_5P_1	7·2	6·8	1·06	5·5	2·58	8·84
B_5P_2	7·3	7·5	0·97	5·2	2·62	9·52
Average	8·39	7·53	1·12	5·5	3·12	8·26

Table VII

Sample 6

Bean plant.	Lamina area in sq. cm. *L.*	Xylem area in sq. cm. × 500 *X.*	Number of vessels *V.*	Average area of vessels sq. cm. × 10^{-4}.	Ratio *X*/*L.*	Ratio *V*/*L.*
B_1P_1	84·80	191·0	336	11·4	2·25	3·96
B_1P_2	73·73	180·9	327	11·1	2·45	4·43
B_2P_1	62·10	181·2	344	10·5	2·92	5·54
B_2P_2	67·03	202·6	338	12·0	3·02	5·04
B_3P_1	55·40	173·7	415	8·4	3·14	7·49
B_3P_2	55·37	167·0	292	11·4	3·02	5·28
B_4P_1	64·27	157·9	248	12·7	2·46	3·86
B_4P_2	66·80	160·1	256	12·5	2·40	3·83
B_5P_1	55·10	125·3	287	8·7	2·27	5·21
B_5P_2	50·80	114·3	243	9·4	2·25	4·78
Average	63·54	168·4	309	10·8	2·62	4·94

Bean plant.	Lamina		Ratio *l*/*b.*	Petiole		*X* as percentage of *P.*
	Length (*l*).	Breadth (*b*).		Length.	Area (*P*).	
B_1P_1	11·1	8·9	1·25	4·5	3·59	10·64
B_1P_2	9·3	9·1	1·02	4·5	2·94	12·31
B_2P_1	9·0	7·6	1·18	6·3	3·75	9·67
B_2P_2	9·2	8·6	1·07	6·5	3·97	10·21
B_3P_1	8·0	7·8	1·03	5·0	3·83	9·07
B_3P_2	7·7	7·6	1·01	4·7	3·32	10·06
B_4P_1	9·9	7·6	1·30	5·0	3·96	7·97
B_4P_2	9·6	8·0	1·20	5·0	4·29	7·46
B_5P_1	8·4	8·4	1·0	5·8	2·94	8·52
B_5P_2	8·3	8·1	1·03	5·6	3·08	7·42
Average	9·05	8·17	1·11	5·3	3·57	9·33

Studies of Growth and Development in the Genus *Fragaria*

III. The Growth of Leaves and Shoot

BY

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With three Figures in the Text

Abstract

Changes in leaf size and in the number and size of the leaf cells have been followed throughout the growing season and during drought and defoliation. The duration of the meristematic phase of primordium growth is the major factor affecting leaf size in all cases, and a hypothesis is developed to account for the changes. A new index of shoot growth reflecting the rate of cell production is used, together with the leaf initiation rate, to distinguish changes in growth rate at the time of runner production and inflorescence initiation.

Introduction

THE mechanism of leaf-size determination has recently received renewed attention, and the relationship between leaf area and cell size has been investigated in *Ipomoea* seedlings (Ashby and Wangerman, 1948, 1950), and in various crop plants, notably sugar-beet (Morton and Watson, 1948). The former investigations were concerned mainly with seasonal and ontogenetic changes, while the latter dealt mainly with the effects of water and nitrogen supply. Similar investigations on the strawberry plant were already well advanced when the account of the behaviour of *Ipomoea* was published, and it seemed important to continue this investigation of a perennial herbaceous plant to provide a comparison with the annual plants already investigated, particularly since Ashby's results pointed to the importance of the age of the plant in leaf development.

In addition it was hoped to throw more light on the relationship between rates of cell division at the stem apex and in the leaf primordia since this relationship is of importance in determining the number of primordia enclosed within the bud (Arney, 1953 *b*); and by integrating the rates of cell division in these two regions an estimate of the total growth of the shoot has been obtained in terms of cell divisions.

Experimental Details

The cultural details have been described elsewhere, and the cell-size and leaf-area data to be considered are for the same plants for which rates of leaf production were obtained (Arney, 1953 *a*).

Measurements were made on the terminal leaflet, which was detached as

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soon as it showed signs of yellowing since removal at an earlier stage might possibly affect the development of younger leaves; the other two leaflets were not removed, even at this late stage, so there is little danger of any effect on the development of younger leaves. After measuring the area of the leaflet a small portion about 2 sq. cm. in area was cut from half-way up one side of the leaflet, dipped in boiling water and decolorized in warm alcohol, and then cleared in hot lactic acid for 5 minutes, and finally mounted on a slide in lactic acid. Epidermal cell size was measured by counting the number of cells appearing in the field of view using a $\frac{1}{8}$-in. objective and an eyepiece specially stopped down. Cells on the periphery which were judged to be more than half included in the field were also counted as whole cells. In order to check on this rough method a more exact method was also used on the same group of cells without moving the slide. This method involved using a camera lucida to trace round the peripheral walls of a group of cells wholly included in the field of view; the area of drawing-paper covered by this outline was determined by cutting out and weighing and comparing with the weight of the whole sheet. The quick approximate method gave a result which was 1 per cent. higher than the more accurate method, based on the average of 68 comparisons; all estimates obtained by the approximate method have therefore been corrected by a factor of 0·99.

The effect of the clearing treatment on cell size was determined by measuring small rectangular pieces of fresh leaflet with a travelling microscope and measuring the same pieces after treatment. There was a net decrease of 2 per cent. in length in both directions (perpendicular to and parallel to the vascular bundles), which indicates a decrease of 4 per cent. in area. Morton and Watson also report a contraction of 4 per cent. (presumably in area). Since the calculation of the number of cells per leaflet involves other approximations and can only be used as a relative index, no correction for this area shrinkage has been made.

A preliminary survey of variations in cell size between positions on the leaflet and successive leaves on a plant was carried out to establish an economical sampling procedure. Measurements of cell area in each of the dozen or so mesophyll islets lying in a straight line connecting two adjacent main veins showed that there was no effect of proximity to the main veins, but other observations showed that islets next to the margin of the leaf had much smaller cells than the rest of the leaf tissue. Measurements of cell area were then made at each of six different positions on nine different leaves produced in May 1950. The six positions were: two adjoining the midrib, two midway between midrib and margin, and two near the margin (but clear of the zone of small cells); one position in each pair was one-third of the way from the apex of the leaflet and the other position one-third of the way from the base. The results showed significant differences due to position and individual leaves:

S.E. (difference between readings on identical tissue) = 128μ^2
S.E. (difference between positions on leaflet) = 80μ^2
S.E. (difference between leaves) = 189μ^2

The existence of significant differences between certain individual plants is established in the appropriate section dealing with the results of the experiments. Since the general object of the experiments was to determine differences between treatments and seasons it is more economical to make a single determination of cell size on one leaf from each plant than to take duplicate readings on any one leaf. The sampling procedure therefore has been to take one leaflet from each individual plant for each sampling occasion and make a single cell count on each leaflet.

Changes in Leaf Size

The seasonal drift in leaflet size for three growing seasons is shown in Table I; the general trend is the same in each case—small leaves produced in early spring and in late autumn, and large leaves during the summer months.

Table I

Seasonal Drift in Leaflet Size: Mean Leaflet Area (in sq. dm.) of Apical Leaflets from Groups of between 6 and 12 Plants for each Month of the Growing Season

	1940	1949				1951
		Plants grown in open border				
	Plants under 15 months old grown in pots in a greenhouse.	(a) under 15 months.	(b) over 15 months.	(c) Dry soil.	(d) Normal soil.	Plants under 15 months old grown in a walled garden.
Feb.	0·19	—	—	—	—	0·13
Mar.	0·33	—	—	—	—	0·16
Apr.	0·46	—	—	—	—	0·27
May	0·41	0·28	0·31	0·31	0·31	0·66
June	0·34	0·33	0·25	0·24	0·30	0·66
July	0·35	0·33	0·32	0·25	0·34	0·67
Aug.	0·51	0·36	0·33	0·21	0·35	0·69
Sept.	0·26	0·26	0·25	0·21	0·26	0·42
Oct.	—	—	—	—	—	0·06
Mean May–Sept.	0·37	0·31	0·29	0·24	0·31	0·62
S.E. of mean	0·02	0·01	0·007	0·01	0·01	0·005

The values for each monthly period are the means of between two and four apical leaflets on each of six or more plants for each season. The most extensive data are for 1951 when 12 plants were used, each contributing over 20 leaves during the season. Analysis of variance shows that the seasonal variation in leaf size is fully significant (1 per cent. level) in each season, and that there are also significant differences between the mean leaf size of individual plants taken over the whole season. In 1949 the statistically significant differences between different plants were only associated with the age of the plants—young plants during their first full growing season (8–18 months old) having significantly larger leaves than plants over 18 months old.

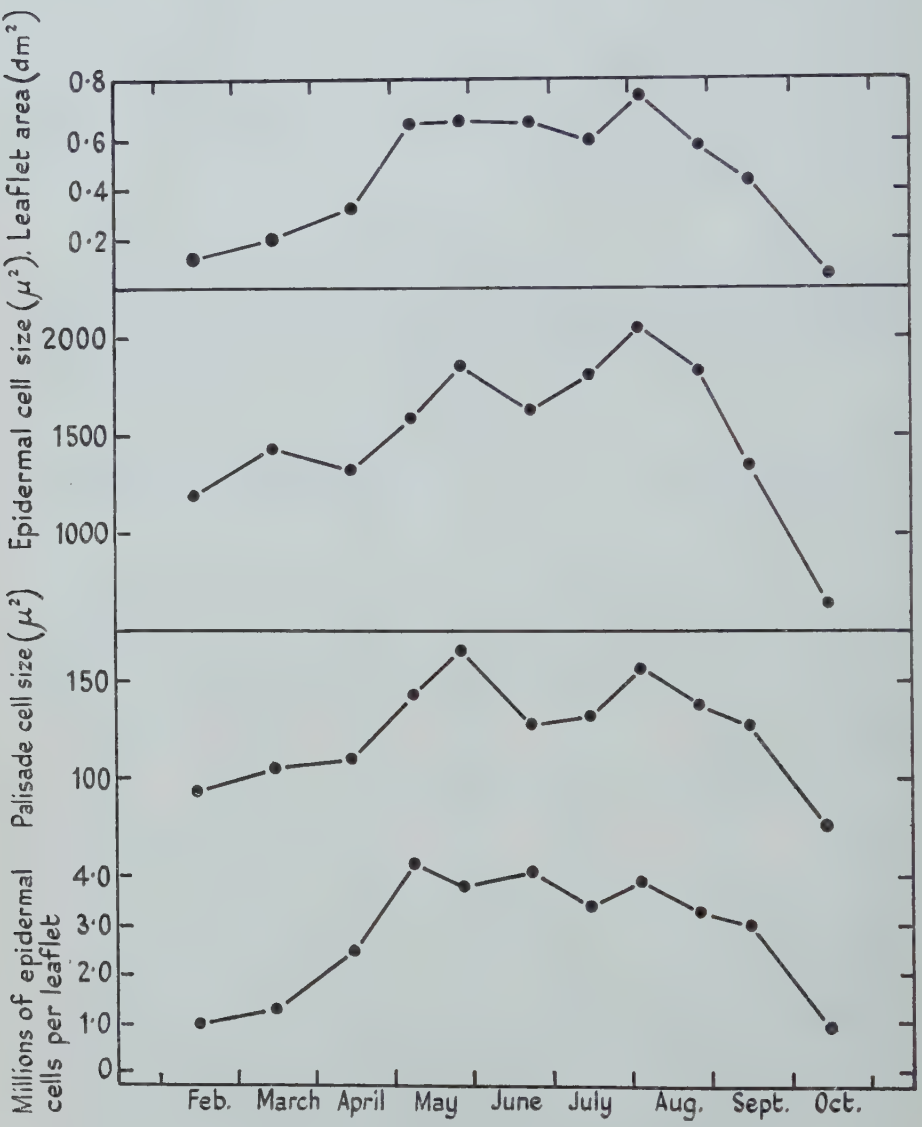
Leaflet area (dm²)
0·8
0·6
0·4
0·2
Epidermal cell size (μ²)
2000
1500
1000
Palisade cell size (μ²)
150
100
Millions of epidermal cells per leaflet
4·0
3·0
2·0
1·0
0
Feb.
March
April
May
June
July
Aug.
Sept.
Oct.

Fig. 1.

This age difference was confirmed in 1950 by Miss O. Gibbon, the mean values for the season being: old plants 0·28 dm.^{2}±0·02 and young plants 0·36 dm.^{2}±0·02.

It has been shown (Arney, 1953) that the drought in 1949 was sufficient to depress the rate of leaf production of plants which were growing in a well-drained situation; these plants also have a significantly lower leaf size (over 20 per cent.) than similar plants growing in more normal soil conditions (see Table I: 1949 dry). All these 1949 plants were transplanted from Southampton to Cardiff in February 1949, and so suffered from disturbed root systems in addition to the dry season; even the plants in normal soil have a leaf size 10 per cent. lower than for comparable plants grown in the same bed in 1950 which were bedded out in the previous August. But this 10 per cent. difference is barely significant, statistically, and the old plants show no difference between the two seasons, so that it can be concluded that dry seasons have little effect on leaf size (a reduction of 10 per cent. or less) unless low rainfall is combined with excessive drainage or poor rooting.

The Relation between Leaflet Area, Cell Size, and Cell Number

In order to determine the mechanism operative in controlling leaf size under various conditions it is necessary to know the relative contributions made by cell size and cell number to differences in leaf area. Seasonal variations, variation between individual comparable leaves, and the effect of drought and of defoliation, have all been studied from this point of view, and it is convenient to describe the results under the four separate headings.

(*a*) *Seasonal variations*

Changes in cell size and in number of cells per leaflet during the 1951 growing season are given in Table II; the values of leaflet area, epidermal cell size, palisade cell size, and number of cells per leaflet are plotted vertically above each other in Fig. 1 to demonstrate the similarity in the seasonal drift of all these quantities. (The leaflet areas in Fig. 1 do not correspond exactly with those of Table I because the latter are based on many more leaflets than were used for cell-size measurements.) The changes in size of epidermal cells and palisade cells are so closely proportional that the epidermal cell size has been taken as a sufficient index of the cell sizes characteristic of all the tissues in a given leaf. Although the seasonal drift in cell size closely follows the drift in leaflet size, variations in leaflet size are not mainly brought about by changes in cell size; the two variables are plotted on a relative basis in Fig. 2 to show the relatively small part played by cell size, which is responsible for less than one-quarter of the total variation in leaflet size, the remainder being due to differences in numbers of cells per leaflet, which would be the result of a

different number of cell divisions during the initiation and development of the primordium.

(*b*) *Differences between comparable individual leaflets*

Successive leaves on any one plant and leaves produced simultaneously by different plants under the same treatment often vary considerably in area.

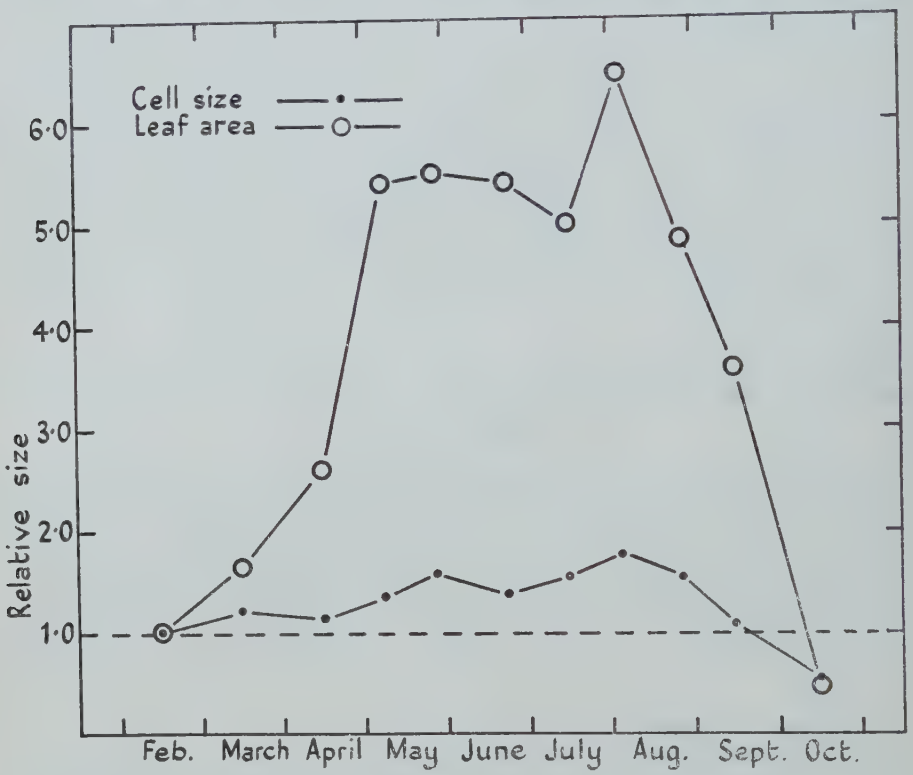

Fig. 2.

Within the groups of twelve leaflets which composed each monthly sample in 1951 there is a close correlation between leaflet area and the number of cells per leaflet, but no correlation between leaflet area and cell size, except in late August and in September. The correlation between leaflet area and cell number is remarkably close (the correlation coefficient ranges from 0·93 to 0·76) from February till June inclusive, but is less close within each month after June. Apparently cell size (vacuolation) may play some part in bringing about variations from the monthly mean during the late summer and autumn, but the number of cells per leaflet is the factor mainly responsible for individual variations in leaflet size.

The variations within each month mentioned above are variations between leaves from different plants, and they will therefore include the effect of

individual differences between the plants (e.g. in root development). Variations between adjacent leaves on the same plant were investigated in 1949, by choosing two leaves from each plant in each month, such that the members of each pair of leaves showed the greatest possible difference in area. Values of cell size for the larger and smaller leaves of each pair were included in separate totals; the 'large' leaflets with a mean area of 1·03 sq. dm. had a mean epidermal cell area of 640 μ^2, while the 'small' leaves with a mean leaf area of 0·66 sq. dm. had a mean epidermal cell area of 600 μ^2. This difference in cell

TABLE II

Cell Size and Number of Cells per Leaflet. Numbers based on Adaxial Epidermal Surface in 1951

Date of leaf initiation.	Date of leaf emergence.	Mean area of one epidermal cell (μ^2).	Mean cross-section area of one palisade cell (μ^2).	Number of epidermal cells per leaflet (in millions).
Late Aug.	Feb.	1,180	94	1·05
Sept.	Mar.	1,430	105	1·39
Early Oct.	Apr. 13–25	1,330	110	2·51
Oct.–Nov.	May 6–16	1,570	142	4·23
Feb.	May 21–30	1,850	166	3·80
Early May	June 22–30	1,620	129	4·10
Mid May	July 11–22	1,800	133	3·38
Early June	Aug. 1–4	2,050	156	3·96
June–July	Aug. 21–28	1,820	137	3·28
July–Aug.	Sept. 8–18	1,350	129	3·04
Early Aug.	Sept.–Oct.	1,270	102	3·14
Late Aug.	Oct.–Nov.	660	75	0·95

size is not statistically significant and would, in any case, only represent 15 per cent. of the total difference in leaflet area. Hence both the variations between comparable plants and between successive leaves on the same plant are almost entirely the result of differences in number of cells per leaflet.

(*c*) *The effect of drought*

The cell sizes and cell numbers in Table III were obtained from some of the 1949 leaves represented in Table I, showing a 20 per cent. reduction in leaf size as a result of low rainfall and exceptionally dry soil. The pooled results for both dry and normal leaflets for July, August, and September 1949 show a very close correlation ($r = 0{\cdot}825$) between cell number and leaflet area, so that the reduction in leaf area induced by drought is mainly the result of lower cell numbers (see also Table III), although there is a statistically significant effect of drought in reducing cell size also. The rate of leaf initiation of the dry plants was appreciably lower than that of the normal plants for the period under consideration, which indicates that cell division at the apical meristem must have been slowed down; it is therefore not surprising that cell division in the leaf primordia should also be affected. Morton and Watson (1948) could detect no effect of water régime on the palisade cell

number in sugar-beet, and although their figures indicate a reduction in palisade cell size under dry conditions, the authors state that this difference was not statistically significant. Ashby (1948) could not detect any difference in epidermal cell size in three strains of *Ipomoea* grown under dry and moist conditions, but the leaves on the dry plants were reduced in size and had correspondingly fewer epidermal cells. The evidence that drought reduces cell size is by no means convincing in earlier work either (see Ashby, loc. cit.), but Table III leaves no doubt that drought does reduce cell size in strawberry

TABLE III

The Effect of Drought on Cell Size and Cell Number. Data obtained from the Abaxial Epidermis in 1949

	Mean area of one epidermal cell (μ^2)		Number of abaxial epidermal cells per leaflet (in millions)	
	Normal.	Dry.	Normal.	Dry.
July .	750	560	4·3	4·2
Aug. .	640	580	5·8	4·3
Sept. .	610	540	4·7	3·4
Mean .	660	560	4·9	3·9

leaves. The reduced number of cells per leaflet in the strawberry in response to drought is in agreement with the results obtained on other plants by Rippel (1919) and by Ashby (1948); it is possible that the absence of any difference in cell number in Morton and Watson's investigations of sugar-beet is connected with some difference in behaviour of the palisade cells, on which they based their observations, and the epidermal cells used in the other cases.

(*d*) *The effect of defoliation*

Two of the plants which showed a statistically significant and consistently lower leaf size for the 1951 season were known to have been almost completely defoliated on May 20, and it seems reasonable to expect that defoliation may have been responsible for the reduction in leaf size, especially since, as shown in Table IV, the two plants both had leaf sizes above the average until the beginning of May. Defoliation had a marked effect on both cell size and cell number, each producing opposite effects on leaflet size and thus minimizing the resulting difference in leaf area. The immediate effect of defoliation is seen in the next leaf to emerge after defoliation, which shows a marked decrease in cell number, and an increase in cell size, compared with the normal plants. By contrast, leaves which emerge subsequently have a lower cell size than normal leaves, the cell numbers returning to normal. The fact that cell numbers return to normal so soon after defoliation seems to indicate that the reduction in cell number is not caused by a shortage of the basic nutrients, since the single remaining emerged leaf (which is still

expanding) could hardly replenish the carbohydrate or protein level of the shoot so quickly.

Morton and Watson (1948) also observed an immediate and very marked (threefold) increase in cell size as a result of defoliation, but in their experiments on sugar-beet there was no effect of defoliation on cell number. Ashby (1948) showed that removal of the two or three partially expanded leaves caused the emerging leaf to develop more cells, and that these were much larger at maturity than they would otherwise have been. Thus the expanding leaves inhibit both cell division and cell enlargement in the emerging leaf.

TABLE IV

The Effect of Defoliation: A Comparison of the Leaflet Area, Cell Size, and Cell Number of Leaves from two Defoliated Plants with the Mean Values for Normal Plants (1951 *Season*)

	Feb. to Apr.	Early May.	*Defoliation* occurred at this time (May 20)	Late May.	June.	July.	Aug.	Sept.
Leaflet area (dm.^{2})								
Defoliated plants	0·29	0·55		0·50	0·63	0·57	0·68	0·30
	0·24	0·37		0·40	0·42	0·56	0·65	0·46
Mean of normal plants	0·20	0·69		0·68	0·66	0·67	0·69	0·42
Epidermal cell size (μ^2)								
Defoliated plants	1,260	1,550		2,020	1,430	1,300	1,630	—
	1,220	920		2,430	1,390	1,250	2,020	1,670
Mean of normal plants	1,310	1,570		1,850	1,620	1,800	1,930	1,350
Cell number per leaflet (*millions*)								
Defoliated plants	2·23	3·54		2·48	3·84	4·30	4·23	—
	1·78	4·02		1·64	3·24	3·68	3·06	3·59
Mean of normal plants	1·65	4·23		3·80	4·10	3·38	3·62	3·04

In Morton and Watson's experiments, and in the case of the strawberry, defoliation included the removal of the expanding leaves, but the results differ in the two cases and both differ from Ashby's results. In all three plants there is a marked increase in cell size, but while *Ipomoea* shows an increased cell number following the removal of the expanding leaves, strawberry shows a marked decrease, and sugar-beet shows no effect at all on cell number.

The Mechanism for Regulation of Leaf Size

The results described in the previous section show that leaf size depends almost entirely upon the number of cells composing the leaf, and that cell size is not the determinant factor, either in seasonal or individual variations, or in changes brought about by drought or defoliation. Differences in the number of cells per leaflet may arise from differences in the number of cells composing the primordium at initiation, or from a different number of cell divisions during primordial development. Evidence will first be presented that differences in

cell number at maturity do not arise from differences in the number of cells in the leaf primordia at initiation.

The mature cell number of leaves initiated in late August is only one-quarter of the mature cell number of leaves initiated in October and November (see Table II); both primordia are enclosed within the apex together at the end of November, the October primordium being almost the youngest and the August primordium the oldest. Anything approaching a fourfold difference in initial size or cell number of the primordia should be reflected in the relative sizes of the primordia throughout all the earlier stages of growth, and would greatly distort the normal size gradation of successive primordia within the bud, which is much the same all the year round. (The size of cells composing the primordia is found to be much the same at all seasons.) The primordial size gradation for November buds was found to be much the same as for other times of the year, the youngest primordia being relatively smaller, if anything, because of the intercalation of inflorescence primordia (Arney, 1953*b*). Hence the October initiated primordia are no larger, and contain no more cells at initiation than the August initiated primordia which produce leaves only one-quarter of the size and cell number at maturity. Similar considerations apply to the leaves initiated in late July and August, which show a threefold drop in cell number per leaf. These primordia will be the four or five primordia which are well developed in the September buds, and one would expect any initial differences in primordium size to act in exactly the opposite way upon the gradation in primordium size in these September buds compared with the November buds just considered. But the primordium-size ratios for August and September buds do not differ from those for the November buds. Hence it seems certain that the final size and cell number of leaves does not arise from any difference in the number of cells composing the primordium at initiation; it follows that cell number and mature leaf size depends mainly upon the number of cell divisions occurring during the development of the primordium.

The absolute rates of various developmental processes change appreciably with season; for example, emergent primordia take nearly twice as long to complete expansion in spring and autumn as they do in midsummer. It is not the absolute rate of cell division, therefore, which is important in determining the mature cell number, but a change in the rate of cell division relative to the rate of other processes determining the time at which cell division stops. Something of the nature of the master reaction which is responsible for the cessation of cell division can be gleaned from the following critical facts which emerge from Table II. There is little difference in cell number between the leaves emerging in May and June although the leaf emerging in early May was initiated in late autumn, while the leaf emerging later in May was initiated in early spring, and leaves emerging in June are initiated only 7 weeks before emergence. This shows that conditions at the time of initiation, including the physiological condition of the apex (which is initiating inflorescences in autumn and is entirely vegetative in the spring), seem to have no effect on

the subsequent development of the leaf primordia. These primordia, which have spent very different periods of time in developing, including winter dormancy in some and not in others, show little difference in final cell number. Hence environmental factors must affect the rate of the master reaction determining the cessation of cell division in exactly the same way as they affect cell division rate, or else the master reaction does not commence until late in primordial development, possibly at emergence. The latter alternative seems to be the only tenable one, for the former would imply that all leaves would have the same number of cells at maturity, under whatever conditions they were developed. Other evidence from Table II points to the same conclusion; leaves initiated in late August and September have a much lower mature cell number than leaves initiated immediately before or after this period. The late August initiated leaves attain almost exactly the same cell number as the early September initiated leaves, although the young primordia develop rapidly in warm conditions at first in the former case, but have to endure the whole winter period in the latter case. Both the late August and September initiated leaves expand and emerge in the cool environment of late autumn and early spring, while the leaves initiated before and after them, possessing much higher mature cell numbers, emerge and expand under warm conditions. Clearly it is the environment at the time of emergence and expansion which controls the number of cells present in the mature leaf, and it must be during this time that the master reaction controlling the cessation of cell division develops.

In a previous study (Arney, 1953*b*) it has been shown that the emergence of primordia corresponds with the onset of rapid vacuolation of the leaf cells and is independent of any influence arriving from either the expanding leaves or the apex and young leaf primordia; nor does it depend upon the breaking of the protective covering of the stipules enclosing the bud. Emergence always occurred when the primordia reached the same critical size. Thus emergence and vacuolation are determined by some master reaction within the emerging primordium itself, and possibly this might be the achievement of a certain cell number. Since emergence always occurs at about the same cell number (= primordium size), and mature cell number may vary over a wide range, the master reaction controlling the cessation of cell division is distinct from the master reaction controlling the incidence of vacuolation. The fact that the removal of the young expanding leaves affects the number of cells in the mature leaf but does not affect the incidence of vacuolation is additional proof of the existence of two separate master reactions for the two processes. The following hypothesis is put forward: The emergence of leaf primordia and the commencement of rapid vacuolation occurs when the primordia achieve a given number of cells which is approximately constant at all times of the year. Cell division may continue after expansion has commenced, and the extent to which late divisions occur will determine the final leaf size, and will depend upon environmental conditions during the period of emergence and expansion. It appears that low temperatures and drought

both inhibit cell divisions during the expansion phase, and thus produce smaller leaves.

Results with *Ipomoea* (Ashby and Wangerman, 1950) show that the leaves in which cell division continues to a later stage have smaller cells; this might be explained on the assumption that the master reaction controlling the cessation of vacuolation acts independently of the master reaction controlling the cessation of cell division, so that the cells of primordia in which cell division continues later will have less time subsequently in which to complete vacuolation. The seasonal variation in leaf size in the strawberry plant is not subject to this type of mechanism, for the small spring and autumn leaves have smaller cells as well as having lower cell numbers. But when the individual variations are considered the situation corresponds to that in *Ipomoea;* there is a highly significant negative correlation ($r = -0{\cdot}55$) between cell size and cell number of individual leaves over the whole period from May to August inclusive, and the correlation within each sample of 12 leaves on any one occasion is much higher ($r = -0{\cdot}99$, $-0{\cdot}83$, $-0{\cdot}87$ respectively for May, July, and August). This is the period when leaves have high cell numbers, and it seems clear that the late cell divisions which are responsible for these high cell numbers do mean that the daughter cells have insufficient time for complete vacuolation. The cool conditions of spring and autumn cause a reduction in mature cell size as well as in cell number. This seems to indicate that the master reactions controlling the cessation of both cell division and vacuolation may be less sensitive to temperature changes than the actual processes of cell division and vacuolation, but more critical information is necessary to distinguish between the various possibilities within this general situation.

Although the mechanism of leaf-size determination in the strawberry may be typical of other plants in which cell division stops at an early stage of leaf development (in the strawberry there is no increase in area of individual leaves later than about 3 weeks after emergence), a different mechanism must apply to plants like marrow-stem kale, in which cell division and leaf expansion continue throughout the life of the leaf (Arney, 1952).

The Seasonal Cycle in Shoot Growth Rate

Shoot growth in the strawberry consists almost entirely in the production of leaves; the main stem elongates only very slowly (about 1 inch per annum) and there is little thickening; the growth of branch shoots is also mainly a matter of leaf formation. The runner internodes produced by a single plant may, together, constitute a considerable bulk of tissue, but most of this growth occurs at growth centres removed some distance from the shoot apex of the parent plant and the runners can therefore conveniently be considered as separate growth units.

The seasonal nature of shoot growth in the strawberry is clearly demonstrated by changes in leaf-production rate during the growing season, but this quantity in itself does not indicate the whole of the seasonal changes in

growth rate, for the leaves produced may vary considerably in size at different seasons of the year. A more comprehensive estimate of shoot growth would be obtained from the total rate at which cells were being produced in the whole shoot; this would require a knowledge of cell numbers in the main stem, petioles, and veins, as well as in the leaf lamina, and in the absence of such information it is necessary to fall back on the assumption implicit in the work of previous investigators, that the cell size of the palisade or epidermis can be taken as an indication of the cell size of the vascular tissue and mesophyll. Accordingly the number of cells per leaflet has been estimated from the formula:

$$N = A(4E+2P),$$

N being the number of cells per leaflet, A the leaflet area, E the number of adaxial epidermal cells per unit area, and P the number of palisade cells per unit area. Information is available that the lower epidermal cells do not differ very much in size from the upper epidermal cells at any season of the year; from inspection a reasonable estimate of the number of mesophyll cells would be twice as many cells as the epidermis. There is a double palisade layer throughout the year.

A typical leaf emerging in August would contain, in each of the three leaflets, 8 million epidermal cells (upper and lower epidermis)+8 million mesophyll cells+104 million palisade cells = 120 million cells in all. It would take 27 'generations' of cell divisions to produce 120 million cells from a single cell, and since the three leaflets and the petiole taken together would contain approximately four times this number of cells (480 million), it seems likely that 29 generations of cell divisions have occurred during the development of the August leaf. This is likely to be an underestimate, since certain areas of the leaf, notably the main veins, cease to be meristematic at a comparatively early stage. Since most of the cell division is completed at about the time of emergence of the primordium the rate of cell division can be calculated from the number of generations of cell division and the time elapsing between initiation and emergence; these rates are given in Table V. Although the rates are only based on approximations, the values are not very sensitive either to wide variations in cell number or to the expected variation in the period of meristematic activity after emergence, so that the cell-division rates which have been calculated can be assumed to be of the right order of magnitude.

The close parallelism between cell-division rate and leaf-initiation rate is expected since leaf initiation is also a result of cell division at the apex. The close connexion between the two has already been demonstrated by other means (Arney, 1953*b*).

An index of cell-production rate has been calculated from the product of the estimated number of cells per leaflet and the rate of leaf production. In calculating this index for each month the cell numbers of the leaves emerging during that month are used, although each leaf primordium is growing for at least 6 weeks, often over 2 months, before emergence. This procedure is

justified because by far the largest number of cell divisions will occur at the end of the meristematic period of each primordium (near the time of emergence); for most leaves almost 98 per cent. of all the cell divisions which occur during the life of the primordium will occur during the last quarter of the meristematic phase.

Monthly values of the index of cell production are given in Table V, and the shoot growth rate is also expressed in terms of the total leaf area produced each month—the product of mean leaf-area and leaf *production* rate (not leaf

TABLE V

Seasonal Growth Rates for 1951

	Number of leaves initiated in 30 days.	Index of cell production rate.	Monthly leaf area production (dm^2).	Number of days between successive cell divisions.*
Feb. . .	0·5	31	0·39	6
Mar. . .	0·7	65	0·72	7
Apr. 13–27 .	1·5	142	1·62	8
May . .	2·1	221	4·2	6
June . .	3·0	358	5·9	2
July . .	3·16	332	6·39	2
Aug. 1–4 .	3·2	386	7·65	2
Aug. 13–20	3·22	—	7·55	—
Aug. 21–28	3·22	322	5·9	2
Sept. . .	3·06	254	3·8	2
Oct. . .	1·68	35	0·3	3

* These are the mean values during the whole of the growth period of the primordia which emerge during the month; they are not the values for growth occurring during the month.

initiation rate). Fig. 3 shows that these two growth indices exhibit the same general trend throughout the season as the leaf-initiation rate. Both cell production and monthly leaf-area production show a marked increase in July when runner production is rising to a maximum, and these high rates are increased in August; the considerable growth of runner plants supported by the parent plant obviously does not depress the leaf growth rate of the parent until late August, if at all. It is not possible to decide whether the drop in cell-production rate in late August and September is an effect of runner growth upon leaf size (as noted by Darrow, 1930) or whether it is the beginning of the autumn decline in growth activity.

The slight decrease in total shoot growth at the end of August, which is indicated by both cell production and leaf-area production, occurs quite definitely before the beginning of inflorescence initiation, and possibly before, or at the beginning of, the induction period; thus the seasonal change in environment at the time of inflorescence induction may have a widespread effect on growth processes of all kinds. Possibly, therefore, the environmental factors operative in flower induction under natural conditions may bring about induction indirectly, through a more general effect on growth; this does not exclude the possibility that the change in growth of the whole apical region of the shoot may bring about the accumulation of a flower-forming

hormone in excess of the threshold quantity. The leaf-initiation rate (which is equal to the leaf-production rate at this time of the year) can be taken as a direct index of the growth rate of the apical meristem itself; this shows only a slight fall, even in September (unlike the total growth of the whole apical region), and this seems to preclude any possibility that a sharp change in the

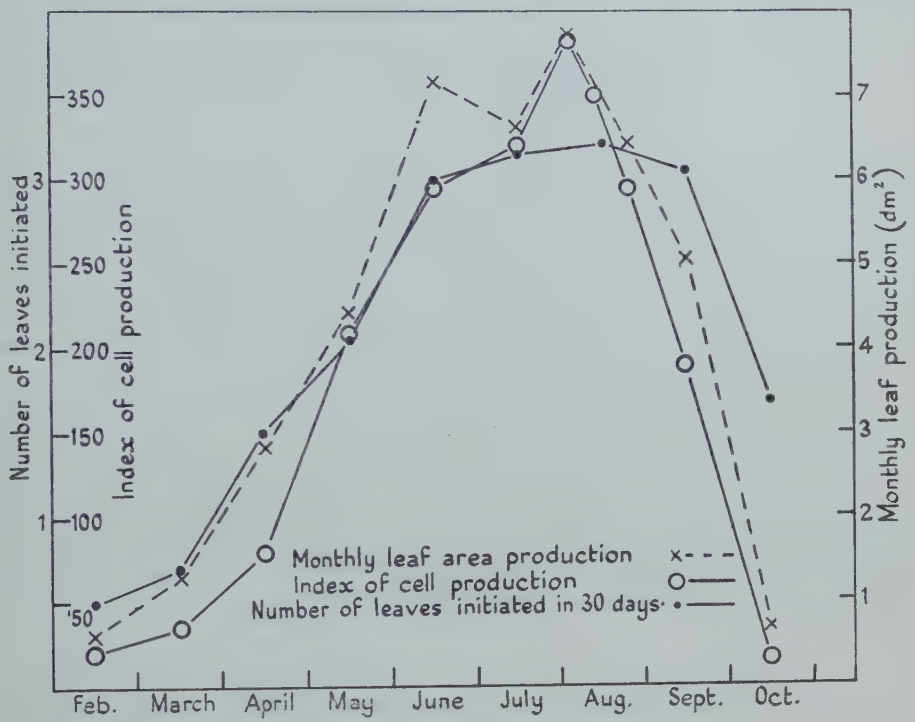

Fig. 3.

growth rate of the apical meristem is a factor precipitating the change to sympodial growth and the transformation of this apical meristem into an inflorescence initial.

In many growth studies it is found that the bulk of the organism studied increases exponentially with time, and the assumption is often made that the amount of growth in any one period depends upon the size of the organism *because* larger organisms can provide greater supplies of metabolites. That this is not the real reason why the amount of shoot growth is linked with the size of the shoot is shown by the defoliation experiments on *Fragaria* and other plants, mentioned on p. 357. In all cases extensive defoliation had only a minor effect on the growth rate of the shoot, although no doubt the dry weight, which is so often used to determine the index of growth, would be affected much more. The real reason for the dependence of shoot growth rate on the size of the shoot is that larger shoots with more leaves have a greater number of loci (the axillary buds) at which cell division can

take place. The importance of the distinction between cell-production rate and the more conventional growth indices can be judged from the difference between Darrow's conclusions about the effect of runnering on the growth of the parent plant and those already outlined above. Darrow showed that leaf area per plant increased exponentially during the spring and early summer until the commencement of runner production, when his growth index

$$\left(\frac{\text{mean daily increment in total leaf area} \times 100}{\text{mean value of total leaf area at beginning and end of period}} \right)$$

began to fall. The fall in this growth index—which is comparable to the relative growth rate of West, Kidd, and Briggs (1920) and the efficiency index of V. H. Blackman (1919)—is taken to indicate that runner production interferes with the growth of the parent plant and competes severely with the parent crown for growth materials. The cell-production rate, which is based on the actual mechanism of plant growth, shows that the rate at which cells are being produced by the parent shoot apex continues to *increase* for at least 6 weeks after active runner production has commenced, and over a period when the growth index based on the exponential method of analysis has been indicating reduced growth rates. The defoliation experiments already discussed show that, even in early May when it would be expected that the flush of spring growth has seriously depleted the food reserves of the plant, the growth of the shoot apex is almost independent of the presence and assimilation of mature leaves. Hence the net assimilation rate too may not always have any relevance to the growth of the plant.

Conclusions

1. There is a considerable change in mature leaf size during the growing season; plants above 15 months old have slightly smaller leaves than younger plants at all times of the year. Individual plants may show consistent differences from normal in leaf size throughout the season.

2. Differences in mature leaf size, whether between individual plants or between successive leaves on the same plant, are not the result of differences in cell size, or rate of cell division, but are caused by differences in the duration of the cell-division phase.

3. Severe drought may reduce leaf size by 20 per cent. or more, and this reduction is mainly the result of an earlier cessation of cell division in the primordium, although decreased cell size also plays an appreciable part.

4. Runner production does not compete with shoot growth of the parent plant; there is an increase in cell production in the parent shoot at the time of runner growth. It is doubtful whether runner production causes any reduction in leaf size; the reduction in leaf size towards the end of the runnering period may well be the beginning of a seasonal decline.

5. There is a slight, but quite definite, drop in shoot growth rates immediately before inflorescence initiation. This drop is shown by cell-produc-

tion rate as well as by the rate of initiation of leaf primordia by the apex, and it probably occurs during the period of floral induction.

6. The mature size to which any leaf attains seems to depend upon the season in which the leaf emerges rather than on the time at which it is initiated. The condition of the apex at the time of initiation and the presence of developing inflorescence primordia do not affect the subsequent development of the leaf primordium. Either low temperatures or drought seem to curtail the period of cell division and reduce leaf size.

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Origin and Development of Axillary Buds in Jute (*Corchorus capsularis*)

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With Plates XVI and XVII

Abstract

The bud initials are laid in the usual manner in the primordial meristem in the case of branching plants of *Corchorus capsularis*. The non-branching character of some varieties is due to the different structural organization of the shoot apex. In the non-branching plants the absence of bud initials is associated with early vacuolation of the meristematic cells. Cases occur of sporadic development of a few axillary and extra-axillary buds.

Introduction

JUTE fibre is obtained from two cultivated species of the genus *Corchorus*—*C. capsularis* and *C. olitorius*. All the varieties of *C. olitorius* and most of the varieties of *C. capsularis* are branched. The branching characteristic is not, however, a desirable character in a bast fibre crop as it reduces the quality. In the normal Indian plants of *C. capsularis* an axillary bud is found invariably in the axil of every leaf. Its size varies from a slight protuberance to a fully developed branch, this being influenced by time of sowing, spacing, and other factors. In some foreign types of this species like 'Jap Red', 'Halmahera', and 'Jap G', obtained from Japan and the Far East, axillary buds are usually absent; in young plants a few buds which are mostly axillary in nature may be occasionally noticeable. Extra-axillary buds develop in all the three non-branching varieties but occur to a greater extent in 'Halmahera'. Unless they are stimulated to grow by insect or accidental damage to the apex, these few buds remain inconspicuous.

The genetics of the branching and non-branching characters has been studied by Patel, Ghose, and Sanyal (1945) and the two characters are found to segregate in a monohybrid ratio, the branching character being dominant over the non-branching. The anatomy of the jute plant has been investigated in detail by Kundu (1942, 1944) especially with reference to fibre formation, cambial activity, and leaf-trace system; no information is, however, available on the structural differences in the organization of shoot apices in the branching and non-branching varieties of jute. It is also not known whether the

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absence of branches in certain types is merely due to bud dormancy or complete absence of bud meristems in the axils of leaves. In order to get this information which is essential in intensive selection work, the developmental study of different *capsularis* varieties was taken up. This paper deals with a comparative study of four varieties of *C. capsularis*, 'D154', 'Jap Red', 'Halmahera', and 'Jap G', differing in branching character.

Materials and Methods

Stem tips of D154, a high-yielding branching variety of *C. capsularis*, were collected to study the different stages of development of axillary buds in the branching variety. Three non-branching varieties of *C. capsularis*, viz. 'Jap Red', 'Jap G', and 'Halmahera', used for breeding purposes, were also used for anatomical study. The non-branching character being (double) recessive, any non-branching plant is pure for that character. Stem tips collected from healthy and vigorously growing plants were fixed in chromic-acetic-formalin mixture and sectioned, using usual microtechniques and cedarwood oil and chloroform for clearing. Sections were cut 8 to 10μ thick. Staining with safranin and fast green yielded better results than other combinations of safranin with haematoxylin or anilin blue.

Observations

The leaves of *Corchorus* are arranged in clockwise spiral in 3/8 phyllotaxy. Phyllotaxy varies from 2/5 to 3/8 according to the vigour of the plant (Kundu, 1944). It has been observed that in both *C. capsularis* and *C. olitorius* both clockwise and anti-clockwise arrangements are present practically in the same ratio. Milliner (1952) has also observed that there is a steady progression with increasing rate of growth to a higher system of phyllotaxis and both types of spirals, dextral and sinistral, occur almost with the same frequency.

In D154 (a branched variety of *C. capsularis*) when the first leaf primordium is very small (being about 30μ in length) it is composed entirely of meristematic cells. Vacuolation starts slightly later on the abaxial side of the second leaf (leaf primordium), while in the third leaf it has extended to the adaxial side also. The central cells of the axis begin to vacuolate strongly at the level of the insertion of the second leaf. Just below the insertion of the third leaf a group of meristematic cells, called 'shell zone' by Schmidt (1924), is delimited among the partly vacuolating cells of the apex (Pl. XVI, Figs. 1 and 2). These meristematic cells are the bud initials, which, by further divisions and growth, give rise to an axillary bud. The 'shell zone' is marked off from the axial meristem by a two-celled layer of vacuolating cells which take fast green after losing some of their safranin stain. Later, however, the bud initials are connected with the axial meristem by 'residual' meristematic cells (Pl. XVI, Figs. 5 and 6). By the time vacuolation starts on the adaxial side of the third leaf, the bud initials are already separated from the axial residual meristem. Appearances thus suggest that they have their origin in the apical meristem and are isolated during vacuolation and growth, as was described

by Wardlaw in *Matteuccia* (1943*a*). By this time also a ring of meristematic cells becomes distinct in the axis, partly due to the vacuolation of the central axial (pith) cells and partly through the vacuolation of the cells on the adaxial side of the leaf which forms the foliar buttress at a lower level. Kundu observed in *C. olitorius* (1942) and later in *C. capsularis* (1944) that the meristem or prodesmogen ring is already interrupted by uniseriate rays at this stage and the cells show radial alinement and are apparently of cambial nature. These cells are included in the term 'residual meristem' (Esau, 1943) adopted here to describe the meristematic region from the time vacuolation starts in the axis (at the level of the insertion of the second leaf) to the insertion of the fifth leaf and to distinguish therefrom 'provascular tissue' used to designate the later stages.

The cells of the axis retain safranin as far as the insertion of the fifth leaf, making it difficult to discern the shell zone, which retains safranin slightly more than the surrounding cells. The shell zone becomes clearer with further enlargement and vacuolation of intervening cells and as it is further separated from the residual meristem of the axis. In the axil of the fifth leaf the shell zone becomes prominent, due to its further expansion and to the contrast in the intensity of staining from the surrounding green cells. The provascular cells can then be seen to extend to the shell zone (in a longitudinal section) and to be continuous from below with the leaf trace above. It is not always possible to distinguish the provascular cells in the meristematic tissue of the bud by the plane of division or shape, but in such cases the red stain has helped in tracing them to the tip of the bud on its ventral side before any bud-leaves make their appearance (Pl. XVI, Fig. 5). The provascular tissue of the bud is always continuous with the provascular tissue of the axis and is hence acropetal in development.

The bud meristem assumes a compact lentil-shaped appearance in the axil of the sixth leaf and the bud separates out (Pl. XVI, Fig. 3). Vacuolation of pith and cortex in the bud starts in the axil of the seventh leaf. In the node of this leaf the bud trace approaches the provascular tissue of the axis as a closed ring. No gap is, however, formed in the provascular tissue. The bud trace, in a longitudinal section of a mature bud, appears as two strands, the upper being continuous with the vascular meristem of the axis and the lower being broken by a leaf gap (Pl. XVI, Fig. 6). As the bud trace merges with the provascular tissue of the axis, the ring is stretched into a loop. At a lower level, leaf gap occurs in the loop of the bud trace. The node is trilacunar. Mitra (1952) reports that the bud traces in *Eupatorium ayapana* and *Jasminus flexile* with decussate leaves form an annulus of procambium below the apical meristem but at lower levels the annulus of procambium becomes two crescentric arcs, whereas in *Morus alba* (alternate 2/5 phyllotaxy) the annulus of procambium becomes an open cylinder of procambium whose two open ends face the bud gap. He states that the bud gap which occurs only in *Morus alba* can be correlated with alternate phyllotaxy. In jute a single gap occurs only for the leaf in spite of the alternate leaf arrangement. In older nodes the

expanding ray cells sometimes give a false impression of bud gap. The leaf gap is considered to arise in the process of extension of the main vascular bundles of the leaves to constitute and organize the vascular system of the main axis (Esau, 1943). The bud has, however, no role in it and so the presence of a separate bud gap is inconsistent with the organization of vascular system.

The first bud-leaf appears in the axil of the eighth leaf. The bud bears two (Pl. XVI, Fig. 4) and four leaves in the axils of the ninth and tenth leaves respectively. The development of the leaves in the bud is acropetal as in the main shoot.

Anatomy of the non-branching varieties

In the adult node of a non-branching plant ('Jap G', 'Jap Red', and 'Halmahera') an axillary bud is usually absent and no shell zone, even in a dormant form, can be detected due to early vacuolation (Pl. XVII, Figs. 7 and 9). Normally the bud initials are not laid down in the axils of the third and fourth leaves as in D154 and they cannot be made out even in the axil of the fifth leaf where they are normally easily distinguished. Rather rarely buds may, however, develop in some axils. This irregular development is seen in all the three varieties, and in such cases the buds seem to originate from the apical meristem as in the normal branching plants. The differentiation of provascular strands could not be studied in successive stages of bud development due to their sporadic development, but in all the cases studied, the bud traces are continuous with the axial provascular tissue.

The buds most often present in unbranched plants arise in a different manner and are extra-axillary. 'Halmahera' is suitable for studying their origin and development. Some apices do not show any development of buds (Pl. XVII, Fig. 7). In such cases shell zones are not seen in the axils of leaves. In addition to the extra-axillary buds, some axillary buds, though rare, develop from the detached meristems in the usual way. When buds develop in the unbranched plants they may be either formed in the axil of the third leaf primordium or else they develop later from the reactivation of previously vacuolated cells of the axis opposite to the fourth to sixth leaf, usually the sixth leaf primordium, probably by diffusion of growth substances from the adjacent inactive detached meristem which is continuous with the apical meristem. Evidently, then, their production is retarded relative to the general development and vacuolation of the apex. Such meristematic cells (extra-axillary) in the axis above the insertion of a leaf are not seen in the branched form D154. Various stages of the development of extra-axillary buds are shown in Pl. XVII, Figs. 8, 10, 11, and 12.

It is also evident that the later development of extra-axillary buds in the non-branching types is slow. The bud meristem can only be detected on the axis 40μ above the insertion of the fourteenth leaf (Pl. XVII, Fig. 11), whereas in the branched D154 the bud emerges from the axil of the sixth leaf. The stage with two bud leaves occurs in the axil of the fifteenth leaf (instead of the

ninth in the D154). Further, all the extra-axillary bud meristems do not develop, but many abort in early stages. Their meristematic cells become vacuolated and lose their continuity with the provascular tissue of the axis (Pl. XVII, Fig. 14). In advanced cases like the bud in the axil of thirteenth leaf (Pl. XVII, Fig. 13) a lenticular mass of parenchymatous tissue remains below it and this retains fast green more instead of safranin as the bud meristem would at that stage of development. Reeve (1943) correlates the early abortion of cataphyllary buds in *Garrya* with the earlier maturation of tissues in the main axis during cataphyll formation. He finds a corresponding sluggishness of meristematic activity in the axils of cataphylls. In 'Halmahera' the case is different; bud initiation is not early in comparison with D154, but their later development is slow, rapidly leading to vacuolation and does not correspond with the growth in the axis.

Discussion

Three types of buds, viz. axillary, cauline, and foliar, have been distinguished on aerial shoots. Although buds in plants from widely different genera have been studied, the number of studies of their ontogeny is barely sufficient to warrant a classification of their modes of development. The classification of buds into axillary, cauline, and foliar types is indeed arbitrary, because the first divisions that mark the initiation of buds occur in the stem so that these and even the leaves are cauline in origin. Rapid divisions and growth take place in the shoot apex, and the bud and leaf initials naturally undergo adjustments in later development. Esau (1953) considers, therefore, that the term 'axillary' is somewhat incorrect as the buds generally arise on the stem but are displaced later. But from the time bud initials are delimited from vacuolating cells either in the shoot apex or the leaf, these show a distinct and characteristic pattern of development. The above classification is, therefore, based on the position that the bud initials happen to occupy at the advent of vacuolation.

Axillary buds arise from a portion of the apical meristem which is detached in the axils during vacuolation and growth (Garrison, 1949*a*, 1949*b*; Gifford, 1951; Krauss, 1948; Louis, 1935; Reeve, 1943). In jute (variety D154) the development of axillary buds follows the normal pattern. These detached meristems in the axils of the leaves usually continue to grow and organize into a bud or may, sometimes, be converted into parenchymatous tissue, as is observed in 'Halmahera' and in *Matteuccia* (Wardlaw, 1943*a*). In the majority of cases the buds grow in the axils of leaves and occasionally show orientation towards the leaf.

Instances of the two other types of buds are few. Detached meristems in *Dryopteris* species (Wardlaw, 1943*b*) as well as in *Matteuccia* (Wardlaw, 1943*a*) and *Onoclea* (Wardlaw, 1943*b*) are situated not exactly in the axil of a leaf but at some distance above the leaf at varying distances. Notwithstanding their extra-axillary position, Wardlaw describes them as axillary, although he admits the incorrectness of the term, because he has not found one more

appropriate. In the unbranched jute 'Halmahera' (in addition to the occasional normal type of bud formation) previously vacuolated cells of the axis above the insertion of leaf which are in continuation of the detached meristem become meristematic and may give rise to buds. The ultimate position of the bud is the resultant of differential activities of stem and bud. The fact that these extra-axillary buds arise usually opposite a leaf indicates an influence of leaf on the bud development (Snow and Snow, 1942). Typical cases of foliar buds developing in a similar manner from previously vacuolated cells of the leaf are reported to occur in *Heracleum* and *Leonurus* (Majumdar and Datta, 1946).

Regarding the nature of the tissue from which buds originate, the interpretations put forward by different authors are conflicting. The origin of the bud from detached meristem is the prevailing condition in plants. The initiation of buds from previously vacuolated cells is rather infrequent (Majumdar and Datta, 1946). The mode of origin does not appear to have any connexion with their position. The mode of origin of buds and differentiation of procambial strands are of special significance in elucidating the organization of the shoot apex. The earlier studies (Hsü, 1944; Kleim, 1937; Rösler, 1928; Sharman, 1945) are confined to investigation whether the buds originate superficially or endogenously since the mode of origin, as Sharman (1945) points out, has special significance in cereal and grass breeding techniques involving the use of colchicine to obtain amphidiploids. Snow and Snow (1942) suggest that axillary buds cannot be initiated unless a leaf or a portion of the subtending leaf primordium is present and infer that the subtending leaf or a portion of it somehow determines the development of bud. Wardlaw (1943*b*, 1946) explains that the buds originate superficially on the shoot in proximity to the meristele conjunction, a region of minimal distension of superficial tissues during growth and development. Ball (1950) gives greater importance to control by the primordial meristem. Wetmore and Wardlaw (1951) and Wardlaw (1952) have reviewed the literature on experimental morphogenesis and connected problems in vascular plants. They conclude that auxin plays a part in the initiation of leaf and bud primordia.

It appears from our results that, in the unbranched jute, vacuolation develops at a much earlier stage or much more rapidly. Apparently this is associated with normal failure of bud development (by detaching fragments of meristem) and such buds as are found are most commonly formed by the reactivation of partly vacuolated cells, which is apparently more difficult to do, as the buds are fewer in number.

The unbranched forms of jute have apparently a recessive gene for this character—hence the processes mentioned (accelerated vacuolation, &c.) are presumably controlled by this gene. Because more rapid vacuolation may have been associated with growth-controlling substances of the auxin type and because this type of substance is known to be involved in the prevention of branching in apical dominance, it is possible that the genic control is exerted through a substance of this type.

Since axillary and extra-axillary buds arise in the apex or very near the apex, these growth-regulating substances seem to be produced by the shoot apex which determines the course of development of buds. The occasional development of buds in the non-branching varieties indicates that the expression of the gene is not complete and other minor or modifier genes are possibly involved and require further study.

Summary

1. The organization of the shoot apex was studied in one branching and three non-branching varieties of *C. capsularis*. Axillary buds develop in the branching variety from a portion of apical meristem detached during growth and vacuolation.

2. Such detached meristems do not exist in the axils of the non-branching plants due to early vacuolation. Some buds develop at irregular intervals, and their development is similar to that in the branching plants.

3. Extra-axillary buds develop by the activity of previously vacuolated cells of the axis which are adjacent to and continuous with detached meristems below. Some fail to develop fully and abort.

4. The non-branching character is controlled by a single recessive gene which probably expresses itself by production of some auxins.

Acknowledgement

We are grateful to Professor W. H. Pearsall, F.R.S., for kindly going through the manuscript.

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EXPLANATION OF PLATES

Illustrating B. C. Kundu and N. S. Rao's article on 'Origin and Development of Axillary Buds in Jute (*Corchorus capsularis*)'.

'Figs. 2, 6, 7, and 8 are taken at a magnification of ×75 and the rest at ×200.

PLATE XVI

Fig. 1. L.S. of shoot apex of D154 showing a shell zone (s.z.) and its continuity with the provascular tissue of the axis.

Fig. 2. Median longitudinal section of D154 showing two bud meristems and a portion of bud.

Fig. 3. T.S. of D154 showing an axillary bud after emergence in the axil of seventh leaf. The bud is in contact simultaneously with the leaf and axis.

Fig. 4. Bud in the axil of ninth leaf.

Fig. 5. L.S. of bud meristem in D154 showing the differentiation of provascular strands (p.s.) on its ventral side.

Fig. 6. L.S. of an adult bud showing its position in relation to leaf and axis and the continuity of provascular strands with the axial tissue. The gap (l.g.) below is for leaf.

PLATE XVII

Fig. 7. L.S. of 'Halmahera' showing absence of bud meristems in the axils of leaf primordia.

Fig. 8. T.S. of 'Halmahera' shoot apex where extra-axillary buds are developing. This section shows two bud traces (B.T.) (residual meristems). One is just merging with the provascular tissue of the axis showing continuity. The other is still separated and is continuous with the extra-axillary bud above.

Fig. 9. T.S. of 'Jap Red' showing absence of buds in the axil of a leaf.

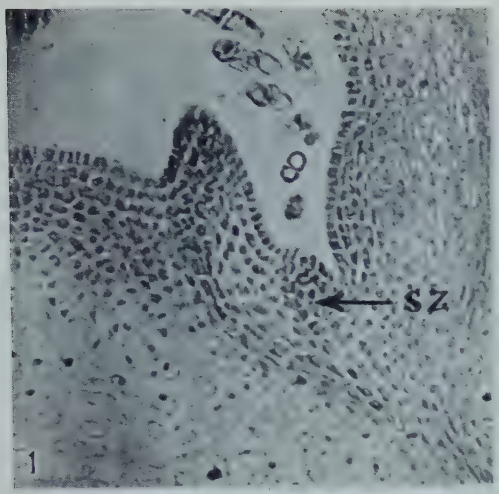
Fig. 10. T.S. of 'Halmahera' showing the development of extra-axillary bud.

Fig. 11. Extra-axillary bud in 'Halmahera' showing its connexion to the axis well above the axil.

Fig. 12. Tangential longitudinal section of an extra-axillary bud showing its position in relation to the axil.

Fig. 13. Aborted bud in the axil of thirteenth leaf in 'Halmahera'. It differs from the one shown in Fig. 10 in staining and lack of bud trace.

Fig. 14. A few sections below the aborted bud showing absence of bud trace and parenchymatization of tissues.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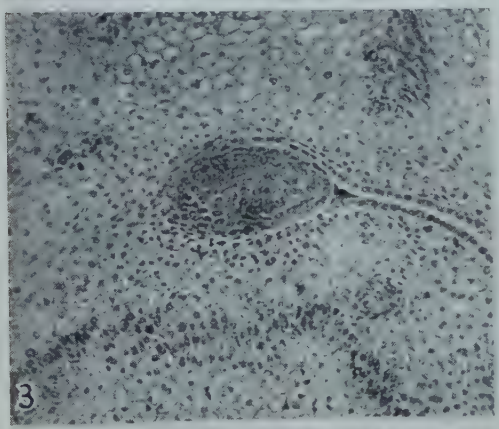
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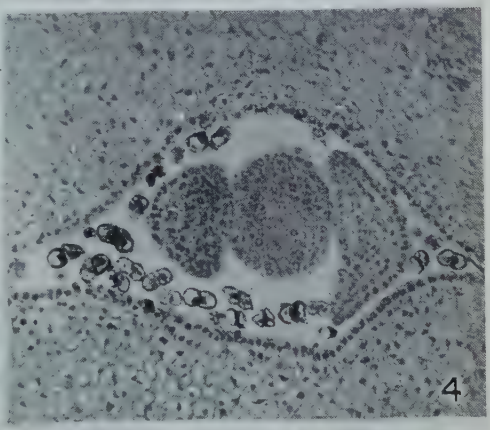
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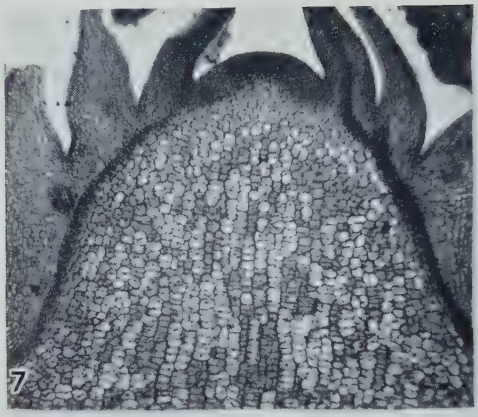
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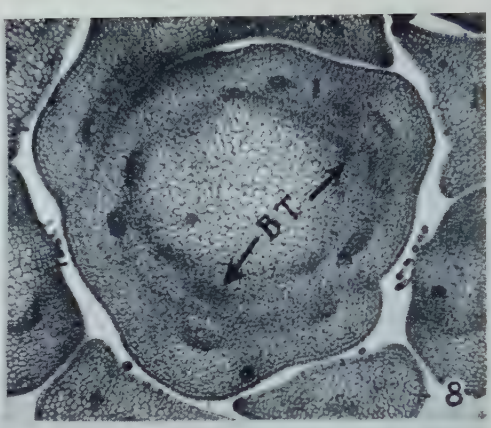
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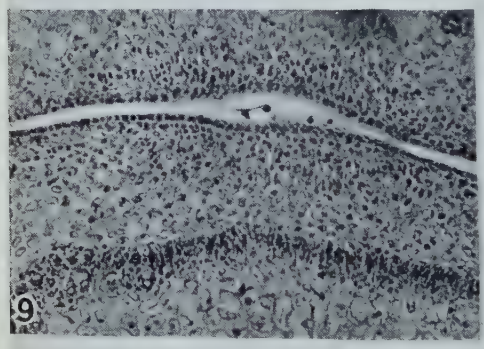
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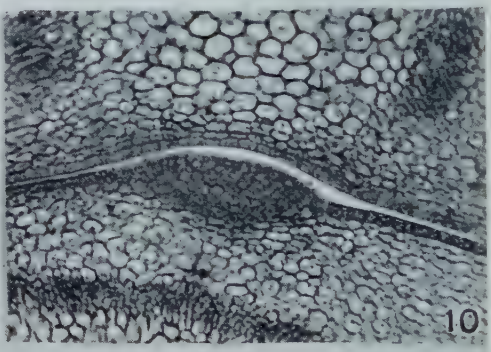
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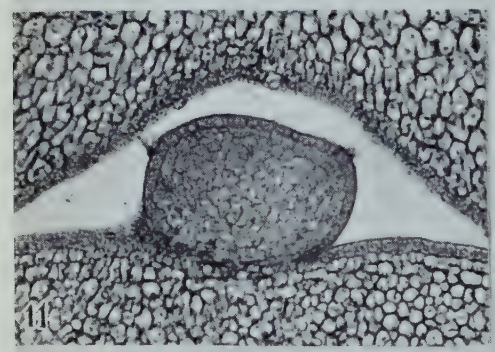
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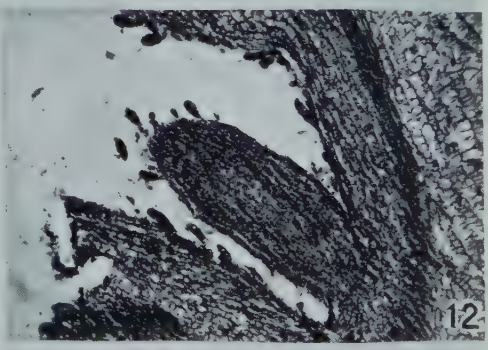
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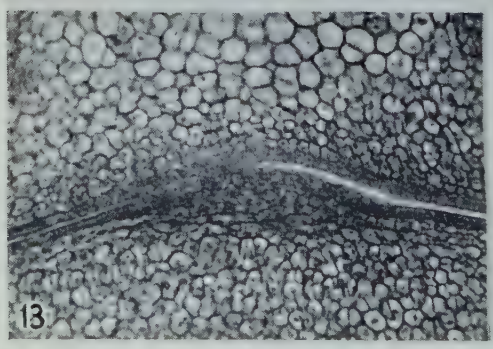
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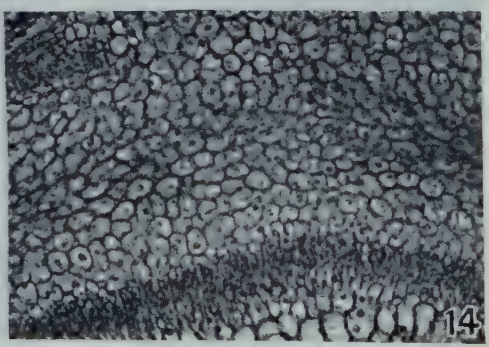
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Induced Apogamy in *Dryopteris dilatata* (Hoffm.) A. Gray and *D. Filix-mas* (L.) Schott emend. and its Significance for the Interpretation of the Two Species

BY

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With Plate XVIII

ABSTRACT

Two new cases of induced apogamy in common European species of ferns are described. Meiosis in the apogamously produced sporophytes has been investigated and compared with that of sexually produced sister plants. The importance of these observations in relation to previous evidence regarding the mode of origin of the wild species is discussed.

THE value of induced apogamy in ferns as a means of obtaining direct evidence on the gametic constitution of a normal species is well known (cf. Manton, 1950), although in the British flora it has so far only been effectively applied to one species, namely the diploid common Hartstongue (*Scolopendrium vulgare* = *Phyllitis scolopendrium*). The reason for this scarcity of records is not far to seek. The method of inducing apogamy by withholding water in the form needed for normal fertilization, though simple and long known (Lang, 1898), is slow. It is also somewhat uncertain. As Lang has shown, many common species if grown under the right conditions and watched for several years will develop some of the earlier stages of incipient apogamy. It is, however, by no means certain that adult sporophytes of any required gametophytic constitution could in fact be raised at will from every wild species. The evidence suggests rather that, while clearly distinguishable from obligate apogamy which manifests itself in very young prothalli without special treatment, induced apogamy varies very greatly in the ease with which it can be brought out, not only from species to species but also among different prothallial strains. We have ourselves had years of experience in working with prothalli of many wild species in extensive breeding-programmes without detecting incipient apogamy as a frequent occurrence in protected prothalli on which controlled fertilizations had not been successful, though such residual prothalli may become very large and bear numerous unfertilized archegonia. In contrast the spectacular manifestations of induced apogamy, such as the cylindrical processes described by Heim (1896) in *Doodia caudata* or the sporangia on prothalli described by Lang (1929) in *Scolopendrium*, attract attention not only by their conspicuousness but by their rarity. If sporophytes

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are raised by sexual means from such prothalli so that a strain becomes established, a repetition of the apogamous phenomena can be obtained fairly easily and without great delay. On the other hand, there are often signs of slight genetical abnormality in the morphology of one or both generations (Lang's *Scolopendrium* had 'ramofurcate' leaves). This suggests that such strains may perhaps be exceptional in having a genetical constitution more favourable than the normal to the apogamous response. Chromosome pairing is not disturbed in such mutant material nor is the specific diagnosis impeded. Their relative rarity among randomly collected wild samples is, however, understandable.

We suspect that this is the reason why not only Lang's *Scolopendrium* but also the two cases to be mentioned here were first detected not as a result of deliberate experiment but as chance contaminants in cultures of other material which attracted attention by conspicuous apogamous developments and were in consequence maintained under observation. The exact wild origin of all three cases is therefore unknown, though the specific diagnoses are simple.

The case of *Dryopteris dilatata* was brought to our notice by Mr. E. Ashby of Manchester who detected it in 1948 and at a later date sent plants to Leeds for cytological study. We are also indebted to Mr. Ashby for the morphological photographs of the early stages and for the historical record, which is as follows.

Early in 1948 a stray prothallus in the roof greenhouse of the Botany Department of Manchester University attracted attention by the presence of grandular hairs and a conspicuous swelling, suggestive of apogamy, on the under side behind the apex. The spore from which the prothallus had come could not have originated in any plant in cultivation in or near the greenhouse, though it could have been introduced either in leaf mould collected in Cheshire or in the tap-water which, in Manchester, is brought from Thirlmere in the Lake District.

By July 1948 a group of sporangia and a small leaf had developed apogamously (Pl. XVIII, Fig. 1). In October the sporangia were detached and mounted in glycerine jelly. They had never opened and could be seen to contain bad spores (Pl. XVIII, Fig. 2). In November 1948 the prothallus was cut into three pieces, one of which still bore the first apogamously produced leaf, and the pieces were transferred to three separate pots which were maintained by watering from below in the usual way.

Two years later, in October 1950, the first prothallial piece had given rise to two apogamously produced sporophytes of different sizes, the larger of which was transferred to Leeds as a small rooted pot-plant not yet fertile. The second prothallial transplant had regenerated copiously, producing numerous apogamous outgrowths bearing little leaves but still without roots (Pl. XVIII, Figs. 3–5). The third transplant had presumably had access to some free water, for the pot was filled with numerous normal-looking sporophytes which suggested self-fertilization. Some of these were sent to Leeds as small rooted pot-plants in 1951.

By 1953 both these and the apogamously produced plant had become fertile and the morphology was sufficiently developed to permit of taxonomic diagnosis. Both are unmistakably *Dryopteris dilatata* (Hoffm.) A. Gray when

TEXT-FIG. 1. *Dryopteris dilatata* (Hoffm.) A. Gray, silhouettes of comparable living leaves taken in 1953 of the sexually produced (left) and apogamously produced plant (right), half natural size.

judged either by their leaf morphology (Text-fig. 1) or by their scale characters, the dark central streak so characteristic of *D. dilatata* in Europe being conspicuous. Both plants are, however, abnormally small, even the larger, supposedly sexually produced specimens being only about half the size usually reached by this species after 3 years' growth. This endorses the suggestion of minor genetical abnormality previously discussed.

TEXT-FIG. 2. *Dryopteris Filix-mas* (L.) Schott emend., silhouettes of comparable living leaves taken in 1953 of the sexually produced (left) and apogamously produced plant (right), three-eighths natural size.

The correctness of Mr. Ashby's diagnosis of the mode of origin was confirmed by the cytological analysis. Pl. XVIII, Fig. 9, obtained from the right-hand plant of Fig. 6, shows 82 chromosome pairs, as is to be expected in normal *D.*

dilatata, which is known to be tetraploid (cf. Manton, 1950). This confirms the sexual origin of the specimen. The apogamously produced plant shows complete failure of chromosome pairing and has only the diploid but not the tetraploid chromosome number, i.e. 82 single chromosomes (Pl. XVIII, Fig. 8). This specimen therefore contains the gametophytic genome of the parent species, as was to be expected. Its significance will be discussed below.

The second case, *D. Filix-mas*, is rather similar, though its origin is less fully recorded. A group of prothalli with large cylindrical processes comparable to those of Heim (1896) were detected in Leeds in the autumn of 1948 in

Text-fig. 3. Explanatory diagram to Pl. XVIII, Fig. 10, to show the arrangement of chromosomes in a meiotic metaphase from the apogamously produced plant of *D. Filix-mas*; paired chromosomes in black, univalents in outline, magnification × 1,500.

an old culture of a different species and a watch was kept upon them. One sexually produced sporophyte had begun to show when the cylindrical processes were first noticed and this plant was maintained as a clue to identity. Two other prothalli by slow degrees developed sporophytes apogamously, which began as a cluster of small leaves as in *D. dilatata* but rooted later. From these, two adult specimens were eventually detached which became fertile in 1952. The sexually produced plant was first fertile in 1953, when the photograph of Pl. XVIII, Fig. 11 was taken. As in the case of *D. dilatata* the development of the sexually produced plant is somewhat retarded, suggesting minor genetical abnormality. The morphology, however, is clearly that of *D. Filix-mas* (L.) Schott sens. strict. (Text-fig. 2), as is that of the smaller, apogamously produced specimen.

Chromosome pairing in the supposedly normal plant is regular, though the quality of preparations is less good than in *D. dilatata* owing partly to scarcity of sporangia. Pl. XVIII, Fig. 11, shows the best cell obtained in 1953, showing the usual 82 pairs characteristic of the species. A comparable stage, from the apogamously produced plant, is shown in Pl. XVIII, Fig. 10, with an explanatory diagram in Text-fig. 3. There are 82 chromosomes, i.e. the gametophytic set, as in the previous case, and almost all of them are unpaired. Unlike *D. dilatata*, however, pairing, though slight, is not completely absent. As shown

in the diagram, there is at least attempted pairing in 5 places in this cell, which is the maximum seen. In other cells the number has varied between 2 and 5, though some pairs are usually very slenderly attached, as shown in the centre of the diagram. This suggests that while there is a little duplicated material among a few of the chromosomes of the *Filix-mas* genome, this is more likely to consist of small duplicated pieces rather than of complete duplicated chromosomes.

Discussion

The facts recorded in the two species under discussion are so similar that both can appropriately be discussed together. In each case we have a parent species which is tetraploid in the normal wild state, and therefore the apogamously produced individuals, though gametophytic in nuclear constitution, are diploid. This distinguishes the two new cases from that of *Scolopendrium* and rather enhances their usefulness.

It may be recalled that the question of an autopolyploid versus an allopolyploid origin had been raised directly or indirectly for both *D. Filix-mas* and *D. dilatata* in Manton, 1950. In both species the absence of multivalents at meiosis is a fact more consistent with allo- than autopolyploidy. In the case of *D. Filix-mas* there was the further evidence from chromosome pairing in the triploid hybrid involving *D. abbreviata*, that the latter diploid species is part-parental to the tetraploid. Comparable evidence has since been obtained for *D. dilatata* (Walker, unpublished). In each, when the tetraploid is back-crossed to a supposedly parental diploid, the resulting hybrid shows n pairs and n univalents. This is strong confirmation of the supposed relationship between diploid and tetraploid, though it is not complete proof since it rests on a plausible though not rigorously tested assumption that the pairs in the hybrid represent the chromosomes homologous with those of the diploid. It could, however, be argued that an autotetraploid crossed with an entirely unrelated diploid would also show n pairs and n univalents, if all the pairs came from the tetraploid species.

This interpretation is improbable because it requires a supplementary hypothesis to explain the absence of multivalents in the tetraploid itself. It cannot, however, be completely ruled out without some additional evidence of the type now supplied by induced apogamy. Had either *D. Filix-mas* or *D. dilatata* been capable of autosyndesis between the two haploid sets of its own genome, this would have been displayed at once by the formation of n pairs in the apogamously produced plants. Since pairing of this type does not occur, the evidence for an amphidiploid origin for both the tetraploid species under discussion is now as strong as it can be made without knowledge of the complete parentage.

This, in our view, is the real importance of the induction of apogamy in species such as these, and the only further progress which can be hoped for would be to find the other diploid species involved in each. Without this we can neither resynthesize the tetraploids nor elucidate the nature of the duplicated

material in *D. Filix-mas*. The latter could mean either a greater measure of homology between the genomes of the original parent species compared with those of *D. dilatata*, or there could have been changes of several different types subsequent to the attainment of the tetraploid state. It is not profitable to discuss this aspect further at present. The main outlines of the situation, however, stand out fairly clearly, namely that in *D. Filix-mas* (L.) Schott emend. and *D. dilatata* (Hoffm.) A. Gray we have two of the best authenticated examples of amphidiploid species now known in the British Flora, although in each case we seem to possess one, but not both, of the diploid component species.

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EXPLANATION OF PLATE XVII

Illustrating I. Manton's and S. Walker's article on 'Induced Apogamy in *Dryopteris dilatata* (Hoffm.) A. Gray and *D. Filix-mas* (L.) Schott emend. and its Significance for the Interpretation of the Two Species'.

Fig. 1. *Dryopteris dilatata*, prothallus with sporangia (marked by white arrow) and an apogamously produced leaf, August 1948. E. Ashby negative 602A. Magnification × 5.

Fig. 2. Detached sporangium from the preceding mounted in glycerine jelly, October 1948. E. Ashby negative 602C. Magnification × 250.

Figs. 3–5. Apogamous regenerations in transplanted pieces of the prothallus of Fig. 1, photographed in August 1950. E. Ashby negatives 854–6. Magnification × 3.

Fig. 6. Two plants of *D. dilatata* photographed in Leeds in June 1953, the apogamously produced plant left, the sexually produced plant right. Magnification × approximately ⅓th.

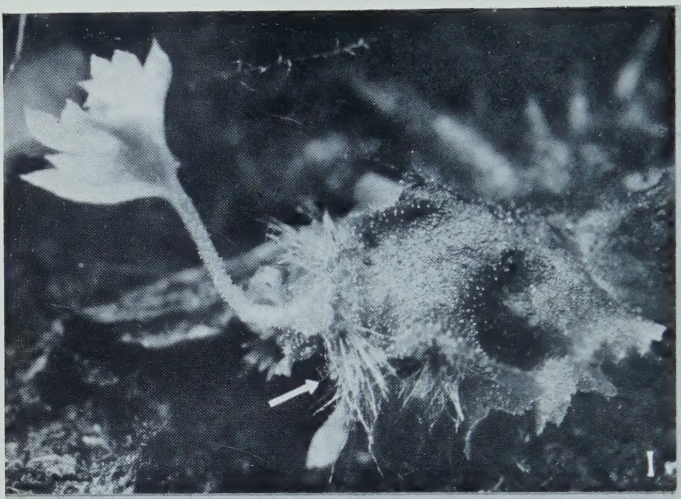
Fig. 7. Two plants of *D. Filix-mas*, other details as in Fig. 6.

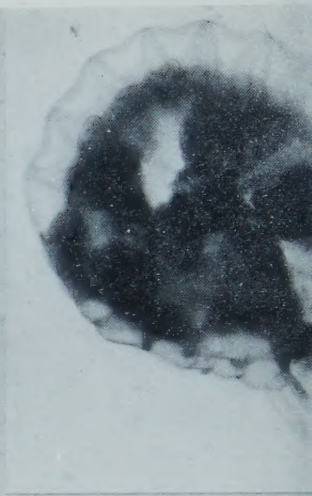
Fig. 8. Meiosis in apogamously produced plant of *D. dilatata* showing 82 univalent chromosomes, from a permanent acetocarmine preparation. Magnification × 1,000.

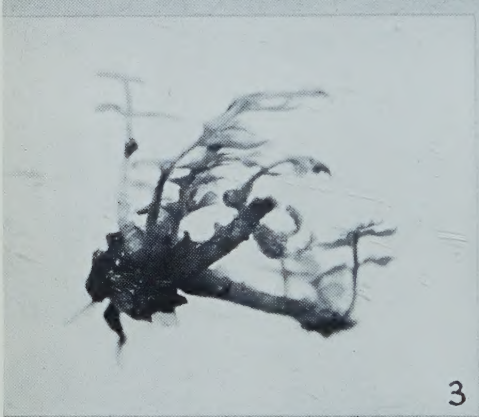
Fig. 9. Meiosis in the sexually produced sister plant to the preceding showing 82 pairs. Magnification × 1,000.

Fig. 10. Meiosis in apogamously produced plant of *D. Filix-mas* showing 82 chromosomes, mostly unpaired. For explanatory diagram see Text-fig. 3 (p. 381). Other details as Fig. 8.

Fig. 11. Meiosis in the sexual sister plant to the preceding showing 82 pairs. Magnification × 1,000.







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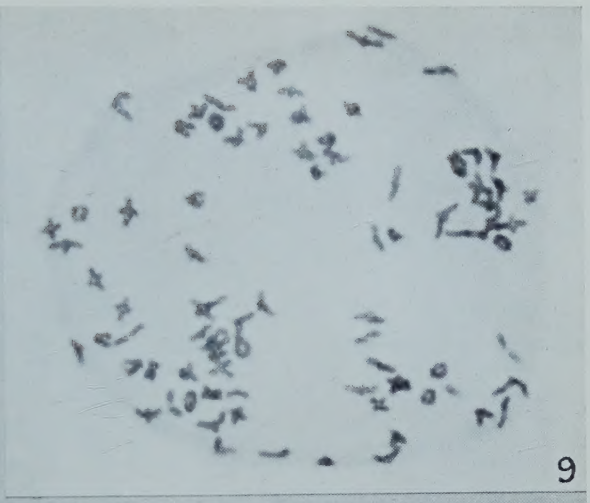
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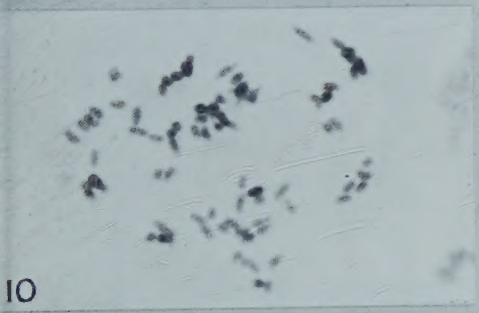
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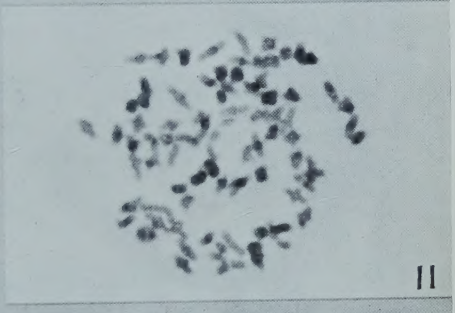
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